

PHARMACOKINETICS OF PLASMID DNA

By

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Brett Edward Houk

This work is dedicated to my parents Nancy and Ronald Houk for all of their guidance throughout my life.

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We sought to construct a complete pharmacokinetic model to describe the degradation of all three topoforms, supercoiled (SC), open circular (OC), and linear (L), of pDNA *in vivo* and *in vitro*. SC pDNA was incubated in isolated rat plasma at 37°C *in vitro*. At various time points, the plasma was assayed by electrophoresis for the amounts of SC, OC, and L pDNA remaining. The calculated amounts remaining were fit to linear differential equations describing this process. The calculated pharmacokinetic parameters suggested that SC pDNA degrades in isolated rat plasma with a half-life of 1.2 min, OC pDNA degrades with a half-life of 21 min, and L pDNA degrades with a half-life of 11 min. Complexation of pDNA with cationic liposomes resulted in a portion of the supercoiled plasmid remaining detectable through 5.5 h *in vitro*. We next investigated the pharmacokinetics of SC plasmid DNA after IV bolus administration in the rat by following SC, OC, and L pDNA. SC pDNA was detectable in the bloodstream only after the highest, 2500 µg, dose and had a clearance of 390(±10) ml/min and volume

of distribution of $148(\pm 26)$ ml. The pharmacokinetics of OC pDNA exhibited non-linear characteristics with clearance ranging from $8.3(\pm 0.8)$ to $1.3(\pm 0.2)$ ml/min and a volume of distribution of $39(\pm 19)$ ml. L pDNA exhibited linear kinetics and was cleared at $7.6(\pm 2.3)$ ml/min with a volume of distribution of $37(\pm 17)$ ml. AUC analysis revealed $60(\pm 10)$ % of the SC was converted to the OC form, and nearly complete conversion of the OC pDNA to L pDNA. Clearance of SC pDNA was decreased after liposome complexation to $87(\pm 30)$ ml/min. However, the clearance of OC and L pDNA was increased relative to naked pDNA at an equivalent dose to $37(\pm 9)$ ml/min and $95(\pm 37)$ ml/min, respectively. We conclude that SC pDNA is rapidly cleared from the circulation. OC pDNA displays non-linear pharmacokinetics. L pDNA exhibits first order kinetics. Liposome complexation protects the SC topoform, but the complexes are more rapidly cleared than the naked pDNA.

CHAPTER 1 INTRODUCTION

Biotechnology is one of the most rapidly growing areas in the pharmaceutical sciences today. However, biotechnology products (e.g. proteins and peptides) suffer from poor stability, low absorption, and difficulties in delivery. It would therefore be ideal if the protein could be made *in vivo*, utilizing the body's own mechanisms to produce the competent protein. Gene therapy is one potential route by which to accomplish this goal. Gene therapy also offers the potential treatment of genetic diseases. The replacement of mutated, missing, or deleted DNA via gene therapy can result in the production of a competent protein. These potentials make gene therapy one of the most exciting and rapidly advancing areas of biotechnology.

Early studies have revealed that systemically administered plasmid DNA (pDNA) can be expressed in animals (Kawabata et al. 1995; Mahato et al. 1995; Osaka et al. 1996; Song et al. 1997; Thierry et al. 1997) and humans (Valere 1999). Intravenous (IV) administration of DNA offers the potential advantage of allowing a wide distribution of activity in the body (Lew et al. 1995; Thierry 1995; Osaka et al. 1996; Thierry et al. 1997). This route of administration allows the treatment of non-localized and systemic diseases. Previous research on the pharmacokinetics of non-viral gene therapies have only been observational citing that plasmid DNA degrades within 5 minutes after incubation in whole blood *in vitro* or after IV injection (Kawabata et al. 1995; Thierry et al. 1997).

Plasmid DNA exists as three major topofoms. The native structure of non-damaged pDNA is supercoiled (SC). Single strand nicks to the phosphodiester backbone of pDNA yield an open circular (OC) form (Figure 1-1). This metabolite of SC pDNA is associated with significant transcriptional activity (~90-100%) (Adami et al. 1998; Niven et al. 1998). Further single strand nicks to the OC pDNA yield linear (L) pDNA, associated with a significant loss of activity (~90%). This process is schematically illustrated in Figure 1-2.

In order to properly dose and achieve the desired levels of gene expression it will be necessary to understand the pharmacokinetics of pDNA. In initial human clinical trials with viral gene therapy, at least one study was terminated due to a patient death (Press 1999). This death was later attributed to the high doses utilized in the trails. Thus, the pharmacokinetics of pDNA is an essential area to be considered as gene therapy approaches clinical use.

The Use of Naked pDNA as a Therapeutic Agent

The use of naked pDNA as a drug after intravenous (IV) administration has been intensely investigated (Wang et al. 1995; Takeshita et al. 1996; Zhang et al. 1997; Budker et al. 1998; Song et al. 1998; Wang et al. 1998; Witzenbichler et al. 1998; Liu 1999; Zhang et al. 1999). The use of naked pDNA *in vivo* was initially reported after intramuscular (IM) or intradermal (SQ) administration in mammals (Wolff et al. 1991; Fazio et al. 1994; Katsumi et al. 1994; Bright et al. 1995; Donnelly et al. 1995; Lopez-Macias et al. 1995; Ulmer et al. 1995; Bright et al. 1996; Corr et al. 1996; Casares et al. 1997; Danko et al. 1997; Lawson et al. 1997; Ragno et al. 1997; Haensler et al. 1999; Noll et al. 1999; Osorio et al. 1999). These studies have definitively shown efficient expression of a transgene can be achieved after administration of naked pDNA. The

successes in these studies suggest that pharmacokinetic modeling of pDNA in the bloodstream after IV administration, or pDNA appearing in the bloodstream after local administration, is an area that must be more clearly defined in order to optimize gene therapy for clinical use.

Effectiveness of Naked Plasmid DNA after Local Administration

Fazio and coworkers (Fazio et al. 1994) demonstrated that a transgene could be efficiently secreted into the circulation after IM administration. Plasma accumulation of human Apo-E2 was demonstrated for at least 45 days after injection. After administration of pDNA encoding for an interferon transgene, interferons were detected from days 7 to 28 post-DNA inoculation (Lawson et al. 1997). Administration of plasmid DNA encoding the chloramphenicol acetyltransferase gene (CAT) in sterile water lead to CAT transgene expression that peaked between 1 and 3 days and was detected up to 28 days after DNA administration. Together these results indicate that sustained expression can be obtained.

Efficient immunization of monkeys, mice, dogs, and cats has been demonstrated using naked pDNA (Katsumi et al. 1994; Lopez-Macias et al. 1995; Ulmer et al. 1995; Bright et al. 1996; Ragno et al. 1997; Haensler et al. 1999; Noll et al. 1999; Osorio et al. 1999). After injection of naked pDNA encoding for influenza hemagglutinin into the skin of mice and monkeys, induction of significant ELISA antibody titers and hemagglutination (HA) inhibition titers that were above the usual threshold values predictive of protection against influenza were demonstrated (Haensler et al. 1999). Mice immunized by various mucosal routes with a pDNA carrying the HA gene (pV1j- HA) induced a HA-specific cytotoxic T lymphocyte (CTL) response. Similarly, nasal

immunization with the DNA vaccine induced primary CTLs against measles virus HA (Etchart et al. 1997).

Plasmid DNA may also serve as an attractive means by which immunization to parasitic infection may be achieved. After injection of pDNA encoding for heat shock protein 65, T cell proliferation and antibodies to this protein were found to be elevated in rats when compared with both an arthritic control and naïve animals (Ragno et al. 1997). A single immunization with pDNA encoding for *Yersinia enterocolitica* 60-kDa heat shock protein (Y- HSP60) was used for vaccination and induced significant Y-HSP60-specific T cell responses after 1 week (Noll et al. 1999). Induction of antibodies against *Salmonella typhi* OmpC porin by naked DNA immunization has also been demonstrated (Lopez-Macias et al. 1995).

A pDNA expression vector encoding human factor IX as an example of immunogen was injected into mice three times at 10-day intervals (Katsumi et al. 1994). This resulted in production of antibodies to human factor IX. Spleen cells from inoculated mice also showed significant cytotoxic T lymphocyte response to target cells expressing human factor IX. Thus, IM and SQ injection of pDNA can induce immune responses against the encoded protein without an exposure to virus particles, and this approach may serve as the basis for immunotherapy in the treatment of cancer and infectious diseases in humans.

Plasmid DNA encoding for viral proteins is also an attractive means by which immunization to viral infection may be achieved. The applicability of pDNA immunization technology for vaccine development was also investigated by immunizing dogs and cats by the IM and SQ routes with a pDNA vector encoding the rabies virus

glycoprotein G (Osorio et al. 1999). The results demonstrated that non-facilitated, naked pDNA vaccines can elicit strong, antigen-specific immune responses in dogs and cats, and DNA immunization may be a useful tool for future development of novel vaccines for these species. Plasmid DNA encoding for the large tumor antigen (T- Ag) of SV40 was used to actively immunize mice to assess the induction of SV40 T-Ag-specific immunity (Bright et al. 1996). Direct injection of the recombinant SV40 T-Ag protein alone failed to induce SV40 T-Ag-specific CTL responses, whereas the pDNA encoding SV40 T-Ag elicited CTL activity specific for SV40 T-Ag. Naked pDNA induced immune responses that were protective against a lethal challenge with SV40-transformed cells.

Naked pDNA has also been successful in the treatment of cancer by local administration. Direct intratumoral injection of free pDNA into mouse melanoma BL6 solid tumor can also result in a high level of transfection. The average amount of chloramphenicol acetyltransferase (CAT) expressed by injecting 30 μ g pDNA containing a CAT gene into a single BL6 tumor was 1.9 $\pm$ 1.0 ng, which is comparable to that reported in the skeletal muscle (Yang and Huang 1996). An intratumoral injection of naked pDNA containing the HSV-TK gene (pAG0) resulted in tumor weight reduction (40-50%) in treated animals versus control groups. Moreover, histopathological analysis on tumors showed large areas of cavitory necrosis (85%) in treated groups compared to controls (10%) (Soubrane et al. 1996). Thus direct injection of free pDNA may offer a simple and effective approach and might be a potential method for cancer gene therapy.

Effectiveness of Naked Plasmid DNA after IV Administration

Naked pDNA administration by IV injection has also been shown to be an effective means by which high levels of gene expression can be obtained (Wang et al. 1995; Takeshita et al. 1996; Zhang et al. 1997; Budker et al. 1998; Song et al. 1998; Wang et al. 1998; Witzenbichler et al. 1998; Liu 1999; Zhang et al. 1999). Budker and coworkers demonstrated that pDNA can be delivered to and expressed within skeletal muscle of rats when injected rapidly, in a large volume (2 to 3 ml) (Budker et al. 1998).

Liu and coworkers also showed naked pDNA can be efficiently expressed in mice (Liu 1999). As high as 45 μ g of luciferase protein per gram of liver could be recovered by a single tail vein injection of 5 μ g of naked pDNA. Approximately 45% of hepatocytes expressed the transgene. Peak expression was obtained at 8 hours after administration and could be retained with repeated injections.

Efficient naked pDNA expression has been obtained following delivery via the portal vein, hepatic vein, bile duct or direct IV administration via the tail vein in mice, rats, and dogs (Zhang et al. 1997; Zhang et al. 1999). The highest levels of expression were achieved after IV administration by rapidly injecting the pDNA in large volumes, approximately 2.5 ml. Over 15 μ g of luciferase protein/liver was produced in mice and over 50 μ g in rats. Equally high levels of beta-galactosidase (beta-Gal) expression were obtained, in over 5% of the hepatocytes that had intense blue staining. Expression of luciferase or beta-Gal was evenly distributed in hepatocytes throughout the entire liver when either of the three routes were injected. Peri-acinar hepatocytes were preferentially transfected when the portal vein was injected in rats. These levels of foreign gene expression are among the highest levels obtained with nonviral vectors. Repetitive

pDNA administration through the bile duct led to sustained foreign gene expression. This study demonstrates that high levels of pDNA expression in hepatocytes can be easily obtained by IV injection.

Takeshita and coworkers (Takeshita et al. 1996) investigated the hypothesis that naked pDNA encoding for vascular endothelial growth factor (VEGF) could be used in a strategy of arterial gene therapy to stimulate collateral artery development. Plasmid DNA encoding each of the three principle human VEGF isoforms (phVEGF121, phVEGF165, or phVEGF189) was applied to the hydrogel polymer coating of an angioplasty balloon and delivered percutaneously to one iliac artery of rabbits with operatively induced hindlimb ischemia. Compared with control animals transfected with LacZ, site-specific transfection of phVEGF resulted in augmented collateral vessel development documented by serial angiography, improvement in calf blood pressure ratio (ischemic to normal limb), resting and maximum blood flow, and capillary to myocyte ratio (suggesting increased vascularization). Similar results were obtained with phVEGF121, phVEGF165, and phVEGF189. This suggests that these isoforms are biologically equivalent with respect to *in vivo* angiogenesis. The potential for VEGF-C to promote angiogenesis *in vivo* was then tested in a rabbit ischemic hindlimb model (Witzenbichler et al. 1998). Ten days after ligation of the external iliac artery, VEGF-C was administered as naked pDNA (pcVEGF-C; 500 µg) from the polymer coating of an angioplasty balloon or as recombinant human protein (rhVEGF-C; 500 µg) by direct intra-arterial infusion. Physiological and anatomical assessments of angiogenesis 30 days later showed evidence of therapeutic angiogenesis for both pcVEGF-C and rhVEGF-C. Hindlimb blood pressure ratio (ischemic/normal) after pcVEGF-C increased after

pcVEGF-C versus controls and after rhVEGF-C versus control rabbits receiving rabbit serum albumin. Doppler-derived iliac flow reserve was increased for pcVEGF-C versus controls and increased for rhVEGF-C versus albumin controls. Neovascularity was documented by angiography *in vivo* after administration of pcVEGF-C and capillary density was measured at necropsy increased. Arterial gene transfer of naked pDNA encoding for a secreted angiogenic cytokine, thus, represents a potential alternative to recombinant protein administration for stimulating collateral vessel development.

Naked pDNA constructs encoding for the human kallikrein protein delivered to spontaneously hypertensive rats via IV injection have been shown to be efficient at controlling hypertension (Wang et al. 1995). The expression of human tissue kallikrein in rats was identified in the heart, lung, and kidney by reverse transcription polymerase chain reaction followed by Southern blot analysis and an ELISA specific for human tissue kallikrein. A single injection of both human kallikrein pDNA constructs caused a sustained reduction of blood pressure, which began 1 week after injection and continued for 6 weeks. A maximal effect of blood pressure reduction of 46 mm Hg in rats was observed 2-3 weeks after injection with kallikrein pDNA as compared to rats with vector pDNA. These results show that direct gene delivery of human tissue kallikrein causes a sustained reduction in systolic blood pressure in genetically hypertensive rats and indicate that the feasibility of kallikrein gene therapy for treating human hypertension should be studied.

Collectively, these results suggest that IV administration of naked pDNA is an attractive means to treat a large range of diseases. However, complete pharmacodynamic modeling of pDNA will have not been achieved. This will allow correlation of the

administered dose with the desired levels of gene expression at the site of activity. Because of the plasmids high molecular weight, anatomical factors must be considered in the movement of these molecules within the body.

Anatomical Factors Potentially Involved in the Pharmacokinetics of Plasmid DNA

Plasmid DNA is a macromolecule having a molecular weight of 3.5 million for a typical plasmid of 5.5 kilobase pairs. This large molecular weight results in an increased likelihood of clearance processes being a function of size and its resulting ability to pass through capillary endothelia. After IV administration, distribution of macromolecules is limited by the structure of the vascular endothelium. The structure of capillaries is diverse among organs. There are 3 main types of blood capillaries: continuous, fenestrated, and discontinuous (Hwang et al. 1997; Takakura et al. 1996).

These 3 types of capillaries are represented in Figures 1-3, 1-4, and 1-5. The diameter of the free plasmid varies from between 8 to 22 nm (Yarmola 1985). The passage of pDNA through a continuous capillary would be limited to the 50 nm pinocytotic vesicles, 2 to 6 nm intracellular junctions, and 50 nm transendothelial channels (Figure 1-3) (Hwang et al. 1997). The basal lamina presents a barrier of collagen, glycoproteins, and fibronectin, macromolecules greater than 11 nm can be retained by the basal lamina. Thus, this may present a barrier for diffusion of the plasmid (Hwang et al. 1997). Continuous capillaries are the most widely distributed in mammalian tissue and are found in skeletal, cardiac, and smooth muscles, as well as lung, skin, subcutaneous tissues, serous membranes, and mucous membranes (Takakura et al. 1996).

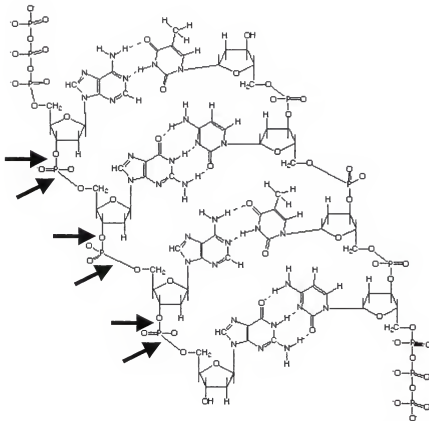


Figure 1-1. Potential sights for nicking of the phosphodiester backbone of DNA.

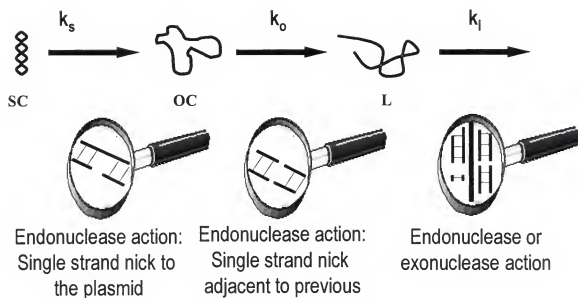


Figure 1-2. Model of plasmid DNA degradation in the bloodstream.

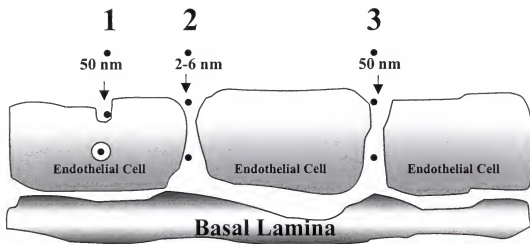


Figure 1-3. Schematic representation of pDNA (•) passing through a continuous capillary: (1) pinocytosis, (2) through intercellular junctions, and (3) passing through endothelial channels.

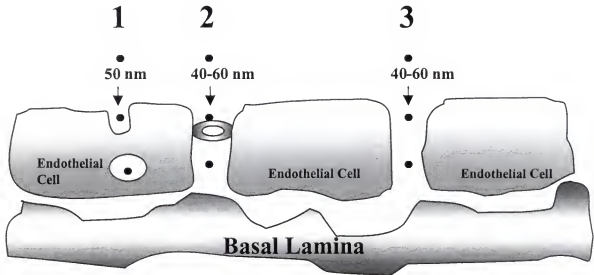


Figure 1-4. Schematic representation of pDNA (●) passing through a fenestrated capillary: (1) pinocytosis, (2) passing through a diaphragm fenestrae, and (3) passing through and open fenestrae.

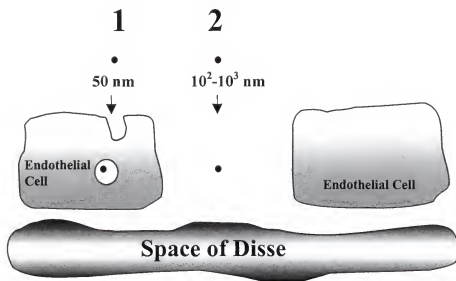


Figure 1-5. Schematic representation of pDNA (•) passing through a discontinuous capillary. (1) pinocytosis and (2) passing through large pores in the endothelium.

Fenestrated capillaries (Figure 1-4) are more likely to allow passage of pDNA into tissues. The pDNA may be transported through mechanisms similar to those involved in the continuous capillary, in addition to transport through 20 to 60 nm fenestrae (Hwang et al. 1997; Takakura et al. 1996). These fenestrae may or may not be closed by a diaphragm. The diameter of the closed diaphragm has not been reported. This type of capillary is generally found in the intestinal mucosa, endocrine glands, exocrine glands, glomerulus, and peritubular capillaries (Takakura et al. 1996).

Discontinuous capillaries are characterized by endothelial gaps and large pores with diameters ranging from 100 to 1000 nm (Figure 1-5) (Hwang et al. 1997; Takakura et al. 1996). In these capillaries there is little restriction of diffusion of macromolecules. Another characteristic of this type of capillary is the lack of a basal lamina (Hwang et al. 1997). The mucopolysaccharide rich interstitial Spaces of Disse have pore diameters ranging from 36 to 50 nm and are unlikely to present a major barrier for the transport of pDNA. The discontinuous capillary is more limited in its distribution than the other types and is found only in the liver, spleen, and bone marrow (Takakura et al. 1996).

These anatomical features can play an important role in the distribution of IV administered pDNA, and other macromolecules. In addition, capillary permeability can be further enhanced in pathophysiological states such as cancer and inflammation (Takakura et al. 1996). Thus the fate of IV administered pDNA is determined not only by physio-chemical properties such as molecular weight, but also by anatomical features of the capillary endothelium present in each tissue.

Degradation of pDNA in the Bloodstream

Early studies suggested that serum nucleases play a major role in the clearance of DNA from the bloodstream of injected animals (Gosse et al. 1965). Investigations by Chused and coworkers suggested that nucleases may not play a major role in the degradation of tritiated KB cell genomic DNA when IV injected in mice (Chused 1972). However, their assay was not able to identify the true activity of nucleases given that their assay utilized genomic DNA. Single strand cuts to the isolated genomic DNA would not yield small fragments and would be undetectable by their method. This would yield an underestimation of true nuclease activity. In contrast, single strand cuts to pDNA would lead to a degradation of the native SC structure to the OC form of the plasmid and be detectable by agarose gel analysis.

Nucleases represent two subclasses of enzymes, endonucleases and exonucleases. Endonucleases act on the phosphodiester backbone of DNA in a continuous chain (Lodish 1995). Whereas, endonucleases act upon the free end (5' or 3') of the phosphodiester backbone in a linear segment of DNA. Investigations by Thierry and coworkers, utilizing agarose gel analysis, suggested that the main nuclease activity in the bloodstream was endonucleolytic. This was based on the finding that the linear to supercoiled ratio increased with time and the SC: OC ratio remained identical to control (Thierry et al. 1997). However this view fails to recognize endonucleolytic activity on linear pDNA also generates degradation products. If endonuclease activity is the primary route of degradation, the kinetic ratios should all remain similar, owing to the fact that exonuclease activity would be masked by endonuclease activity. The pharmacokinetics of this degradation remain to be determined and may serve as a valuable tool in the understanding of the mechanisms of pDNA degradation observed in the bloodstream.

Pharmacokinetics of Liposomal Delivery Vehicles

Although few studies are available on the pharmacokinetics of liposome:pDNA complexes, liposomal pharmacokinetics alone have been studied extensively with several reviews published (Hwang et al. 1997; Juliano 1988; Takakura et al. 1996). Liposome pharmacokinetics have been shown to be dependent upon size (Sato 1986), dose (Bosworth 1982; Osaka et al. 1996), lipid composition (Gabizon 1988), and charge (Juliano 1988). In general, liposomes larger than 60 nm in diameter are unable to access tissues having continuous capillary endothelia, including skeletal, cardiac, and smooth muscle, lung, skin, subcutaneous tissue, and serous and mucous membranes, and are limited to uptake in tissues of the reticuloendothelial system (Hwang et al. 1997). Liposomes larger than 0.5 μm are confined to the vasculature in all tissues.

Pharmacokinetics of Naked Plasmid DNA and Liposome: Plasmid DNA Complexes

After systemic administration of pDNA alone or as liposome:pDNA complexes, DNA rapidly disappears from the bloodstream. The processes responsible involve degradation in the blood stream, interaction with plasma proteins, organ distribution, and uptake by the reticuloendothelial system. The transport of DNA and liposome:pDNA complexes into organs is roughly a unidirectional system, where distribution back into the central compartment can be assumed to be negligible (Mahato et al. 1997).

Pharmacokinetics of Naked Plasmid DNA in the Blood after IV Injection

Plasma levels of pDNA may be measured using radiolabeled DNA or agarose gel analysis (Kawabata et al. 1995; Lew et al. 1995; Mahato et al. 1995; Osaka et al. 1996; Thierry et al. 1997). Using agarose gel analysis, Thierry and coworkers found that SC plasmid DNA is not detectable in either murine plasma or cell fractions 1 minute after injection of naked plasmid DNA in mice (Thierry et al. 1997). OC and L forms have

been detected through 30 minutes post-injection by Southern blot analysis (Lew et al. 1995). The half-life of intact (OC or L) plasmid DNA is less than 5 minutes. Degraded plasmid fragments remain detectable in the blood at 30 minutes post injection. By 60 minutes even degraded plasmid is cleared. This elimination has been shown to be independent of the DNA sequence (Lew et al. 1995).

When plasmid DNA was administered to mice in the form of liposome:pDNA complexes, SC DNA was detected in the blood between 1 and 60 minutes after injection (Thierry et al. 1997). OC DNA degrades with a half-life of approximately 10 to 20 minutes. Uptake of pDNA in blood cells reaches a maximum as early as 1 minute after injection of liposome:pDNA complexes.

A major problem associated with these studies is that the analysis of samples was done only qualitatively. No attempt was made to quantitate the amounts and types of plasmid present in the bloodstream at various times. This information is critical for an evaluation of the predictive value of pharmacokinetic parameters associated with gene delivery.

Quantitative analysis of gene delivery has been done using IV injected radiolabeled pDNA, [^{32}P] or [^{33}P], in mice. In these studies, the half-life of naked pDNA is approximately 10 minutes (Kawabata et al. 1995; Osaka et al. 1996). The total plasma radioactivity displays a degradation pattern consistent with a two compartment body model (Mahato et al. 1995). Total body clearance of naked pDNA is estimated at 102 ml/hr, and plasma AUC is estimated at 0.98 (% of dose*hr/ml). Urinary radioactivity increases with time, indicating the degradation products are excreted via the kidney. Similar results were obtained following injection of radiolabeled liposome:pDNA

complexes with AUC's of 0.57 to 0.7 (% dose*hr/ml) and total clearance ranging from 175.8 to 142.7 (ml/hr) (Mahato et al. 1995). Half-life for radiolabeled pDNA: dimethyldioctadecylammonium bromide: dioleoylphosphatidylethanolamine complexes was shorter ranging from 4 to 8 minutes (Osaka et al. 1996) suggesting rapid tissue entrapment of the liposome:pDNA complexes relative to naked plasmid. Twenty-four hours after injection, blood cell and plasma radioactivity for naked pDNA and liposome:pDNA complexes were similar (Osaka et al. 1996).

Between these 3 analysis methods (agarose gel, Southern blotting, and radiolabeling) agarose gel analysis can determine more detailed information on the degradation of different structures of plasmid, (SC, OC, and L). This method is easy to apply and can be done under normal conditions without the limitations associated with radioactivity. The disadvantage of this method is that it is traditionally a semi-quantitative method. The advantage of the [^{33}P] and [^{32}P] methods is that these are quantitative methods and are more sensitive than agarose gel analysis. The disadvantages are that radiolabeling yields OC pDNA and thus, this method gives no information on the pharmacokinetics of SC pDNA. OC plasmid can also not be differentiated from L pDNA. The radioactivity is also counted without discriminating the degraded DNA fragments or the free label. Furthermore, special conditions and precautions are needed to handle radioactive materials. The difference between [^{32}P] and [^{33}P] is that [^{33}P] has less personal danger and offers greater ease of handling than [^{32}P] (Song et al. 1997; Niven et al. 1998).

Overall, when pDNA is injected in mice, SC pDNA has not been detected when administered as naked pDNA, but is after the injection of liposome:pDNA complexes.

After administration as naked pDNA, OC and L pDNA degrades with a half-life of between 5 and 10 minutes. The half-life of OC or L pDNA after administration of liposome:pDNA complexes ranges from 4 to 20 minutes. OC pDNA is available for transcription if taken up by cells. (Adami et al. 1998; Niven et al. 1998) Thus, administering pDNA in the form of liposome:pDNA complexes may offer a slight increase in the availability of IV administered pDNA.

Distribution of Plasmid DNA in Tissues after IV Injection

Tissue distribution of pDNA may be measured using radioactivity, Southern analysis, or whole body autoradiography. Using Southern analysis, pDNA has been detected in the bone marrow, heart, kidney, liver, lung, spleen, and muscle as early as 1 hour after injection (Lew et al. 1995; Niven et al. 1998). No plasmid was detectable in the brain, intestine, and ovaries.

Sub-picogram levels may be detected using polymerase chain reaction (PCR). Using this method, Lew and coworkers showed that at 7 days after IV injection, the range of residual plasmid was 1 fg/ μ g in the brain, intestine, and gonads, and was 64 fg/ μ g in the marrow, heart, liver, spleen, and muscle (translating to approximately 250-16,000 copies/genome (Lew et al. 1995). By 28 days post-injection, levels of detectable plasmid had decreased 128 fold. Using PCR, residual plasmid remained detectable 6 months post injection at 2 to 8 fg/ μ g genomic DNA and was predominantly in the muscle.

After injection of radiolabeled plasmid, distribution may be measured by isolating tissues and measuring homogenates in a scintillation counter (Kawabata et al. 1995; Mahato et al. 1995). Alternatively, the entire carcass may be measured by whole body sectioning and autoradiography (Osaka et al. 1996; Niven et al. 1998).

After injection of radiolabeled naked pDNA, accumulation of radioactivity occurs initially in the lung, but declines rapidly through 1 minute post-injection (Kawabata et al. 1995). Osaka and coworkers found that by 2 minutes after injection of naked pDNA, organ distribution is liver>spleen>lung, blood (Osaka et al. 1996). Whereas, Niven and coworkers found the time to reach maximum levels in the lungs is as long as 5 minutes versus 2 hours in the liver (Niven et al. 1998). Thus, there appears to be an initial rapid entrapment and transient accumulation in the lungs with accumulation occurring in the liver after a short period of time. Plasmid DNA was preferentially recovered in the non-parenchymal cells in the liver suggesting that the liver is acting in a scavenger role in uptake (Kawabata et al. 1995).

When compared to naked pDNA, IV injection of liposome:pDNA complexes shows a higher accumulation of radioactivity in the lung 2 minutes after injection, Osaka and coworkers showed the major organs exhibit a distribution of lung>liver>spleen>kidney (Osaka et al. 1996). One hour after injection, a slight rise is seen in most organs, which is probably related to continuous uptake by the reticuloendothelial system. By 24 hours after injection of liposome:pDNA complexes, lung radioactivity dropped approximately 70 fold, with a distribution in major organs of spleen>liver>lung, kidney.

Conclusions

A complete understanding of the classical pharmacokinetic parameters of gene delivery is necessary to move genetic agents forward as clinical therapeutics. Problems include the rapid clearance of naked pDNA and liposome:pDNA complexes without expression of the gene products, poor target tissue specificity, and degradation in the plasma. After systemic administration in mice, plasmid DNA is rapidly eliminated from

the circulation by extensive uptake by the reticuloendothelial system and degradation by plasma nucleases. Hepatic uptake is almost identical to liver blood flow suggesting highly efficient uptake. A complete pharmacokinetic model of all 3 forms of plasmid DNA (SC, OC, and L) has not been proposed. As the products of the biotechnology industry begin to move towards more clinical applications, the pharmacokinetic modeling of gene delivery will likely become an intensely investigated area.

CHAPTER 2 PHARMACOKINETICS OF PLASMID DNA IN ISOLATED RAT PLASMA

Introduction

In vivo delivery of plasmid DNA (pDNA) encoding for therapeutic proteins to patients via parenteral administration is an attractive means by which to target the gene to a wide variety of tissues. Early studies revealed that endogenous enzymes present in the plasma play a role in the clearance of nucleotides from the bloodstream (Chused 1972; Whaley 1972; Chia 1979; Piva 1998). These early studies have displayed that pDNA incubated in the presence of 10 % fetal bovine serum shows initial degradation by 15 min and is completely degraded by 60 min (Piva 1998). Similar results have been displayed in the presence of 90% human serum (Piva 1998).

Nucleases will convert the native supercoiled (SC) pDNA topoform to the open circular (OC) and linear (L) forms of the plasmid (Lodish 1995). Changes in topoform have been associated with alterations in transcriptional activity. The significance of this change has been the matter of some debate. For example, the OC form of the plasmid has been shown to express similar levels of chloramphenicol acetyl transferase and luciferase proteins (Adami et al. 1998; Niven et al. 1998) to 2 to 4 times less (Hirose 1993; Cherng 1999; Ramsey 1999) levels of transcribed luciferase and lac-Z, proteins. Increases in the amount of supercoiling serves to further increase the percent maximal transcription (Ramsey 1999). Furthermore, the time required for formation of the transcription preinitiation complex has been shown to be decreased with a SC template (Hirose 1993). Degradation to the L form of pDNA is associated with significant losses

in transcriptional activity (90-100%) (Hirose 1993; Adami et al. 1998; Niven et al. 1998; Cherng 1999; Ramsey 1999). Differences in transcriptional activity may need to be accounted for in future pharmacodynamic studies.

Early studies revealed that serum nucleases play a role in the rapid clearance of genomic DNA from the circulation of injected animals (Gosse et al. 1965). Recent studies on the pharmacokinetics of pDNA have attempted to use radiolabeled pDNA for detection (Osaka et al. 1996; Niven et al. 1998). However, the radiolabeling procedure involves nick translation, thereby eliminating the possibility of maintaining the SC topoform. Furthermore, this method does not discriminate the degraded pDNA from the intact plasmid, thus yielding an overestimation of the true half-life of the intact pDNA. Other studies on the pharmacokinetics of SC and OC pDNA have been only qualitative citing the presence of pDNA topoforms at various time points (Kawabata et al. 1995; Osaka et al. 1996; Thierry et al. 1997).

Thierry and coworkers studied the stability of pDNA in the bloodstream of mice after IV injection (Thierry et al. 1997). Their results indicated that SC plasmid was not detectable in the plasma or red blood cell fractions 1 min after injection of pDNA. The true half-life was unable to be calculated using their method due to this rapid degradation. Kawabata and coworkers found that the SC pDNA was completely converted to the OC topoform within 5 min when incubated in mouse whole blood (Kawabata et al. 1995). Little other information on the pharmacokinetics of pDNA is available. The exact pharmacokinetics underlying this rapid degradative process is not fully understood. To properly dose and reach the desired therapeutic endpoints, a thorough understanding of the pharmacokinetics of pDNA is a necessity.

It is necessary to study the effects of plasma on pDNA in order to begin understanding the importance of the degradation of pDNA in the blood and allow a foundation upon which comparisons of delivery vehicles can be made. Naked pDNA has been shown to remain in the plasma fraction of blood (Osaka et al. 1996). For these reasons, we sought to investigate the pharmacokinetic processes underlying the stability of pDNA in a rat plasma model. We further sought to construct a complete pharmacokinetic model to describe the degradation of all three topoforms of pDNA in plasma. This model will allow a prediction of the time course of potential tissue exposure to the transcriptionally active SC and OC pDNA topoforms.

Methods

Phenol: chloroform: isoamyl alcohol (25: 24: 1, v/v/v), Tris, boric acid, EDTA, and agarose were purchased from Sigma Chemical Company (St. Louis, MO). Ethidium bromide (electrophoresis grade) was purchased from Fisher Biotech (Fair Lawn, NJ). Competent JM109 bacteria (Promega, Madison, WI) were transformed according to the manufacturers directions with the pGL3 control plasmid (Promega, Madison, WI), pGeneMax-Luciferase (Gene Therapy Systems, San Francisco, CA) or pGE150 plasmid (a generous gift of Dr. G. Elliot, Marie Curie Research Institute, The Chart, Oxted, Surrey, UK). Representative plasmid maps are presented in Figures 2-1, 2-2, and 2-3 for the pGL3, pGeneMax-Luciferase, and pGE150 plasmids, respectively. Plasmid DNA was isolated from overnight cultures using the Plasmid Maxi-Prep kit (Quiagen, Valencia, CA), and was >95% SC by agarose gel analysis.

Blood was isolated from male Sprague-Dawley rats (300-350 g) by cardiac puncture, and immediately placed in heparinized test tubes (Vacutainer, Becton

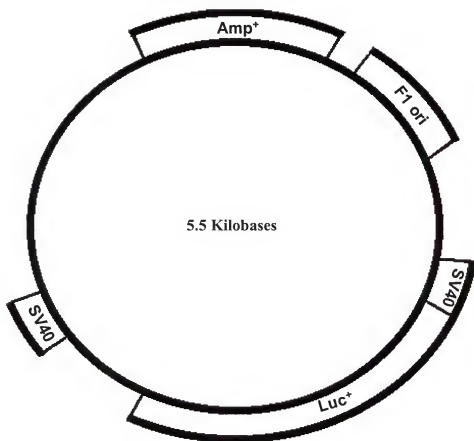


Figure 2-1. Plasmid map of pGL3 Control.

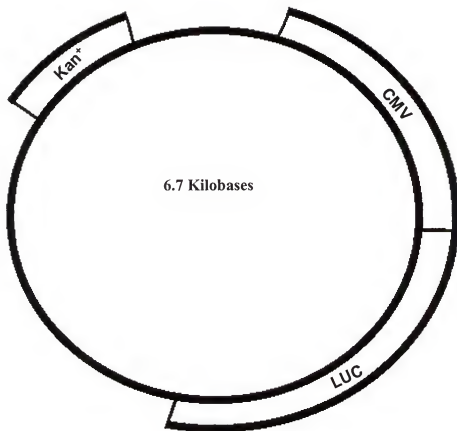


Figure 2-2. Plasmid map of the pGeneMax-Luciferase.

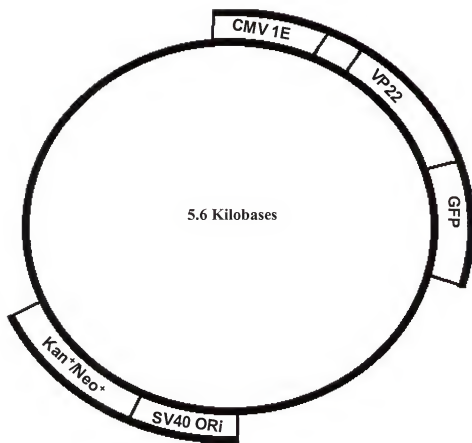


Figure 2-3. Plasmid map of pGE150.

Dickinson, Franklin Lakes, NJ) on ice at the times indicated. Blood samples were centrifuged at 6,000 g for 5 min. For dilution experiments, plasma was diluted to 25 and 50% with PBS or PBS containing 0.1 mM EDTA. To analyze the effects of heat, plasma samples were incubated at 90°C for 10 min in sealed tubes before assay. Plasma (600 µl) was removed and placed on ice until assay. Plasma samples were warmed to 37° C in a water bath and maintained at 37° C for the duration of the experiment. Plasmid DNA (12 µl/ 17 µg) in TE buffer was incubated in the 37° C plasma and 50 µl samples were taken at various times. Phenol: chloroform: isoamyl alcohol (25: 24: 1, v/v/v) (80 µl) was immediately added to each sample, vortexed for 5 s at low speed, and placed on ice. Samples were centrifuged at 20,800 x g for 10 min at room temperature. From the supernatant, an aliquot of 15 µl was removed, 5 µl of 6 x loading dye (Promega, Madison, WI) added and placed on ice until loaded on an agarose gel.

Samples were loaded on 0.8% agarose in 0.9 M Tris-Borate and 1 mM EDTA gels containing 0.001% ethidium bromide. Electrophoresis was carried out at 0.30 V/cm³ for 12 h. The ethidium bromide was excited on a UV light box (Model TFX-35M, Life Technologies, Grand Island, NY) and the net fluorescence intensity captured at 590 nm on a Kodak DC120 digital camera (Eastman Kodak, Rochester, NY). The amounts of SC, OC, and L pDNA were calculated using Kodak Digital Science 1D Image Analysis Software (version 3.0, Eastman Kodak Company, Rochester NY) with a Lambda Hind III digest (Promega, Madison WI) and a High DNA Mass Ladder (Life Technologies, Grand Island, NY) as external standards. Accuracy was 96 to 104% for SC pDNA, 98 to 108% for OC, and 96 to 113% for L pDNA. Percent coefficient of variation was ≤ 5%, 19%, and 13% for SC, OC and L forms of the plasmid respectively. Standard curves for all

three forms of the plasmid (Figures 2-4, 2-5, and 2-6) were linear between 10 and 250 ng pDNA bands ($R^2 = 0.9995, 0.9985, \text{ and } 0.9933$ for SC, OC, and L respectively). All reported concentrations were calculated from bands within the range of the standard curves. Lower limit of quantitation was 0.5 ng/ μ l. for all three forms of the plasmid. Lower limit of detection was 0.25 ng/ μ l for all three forms of the plasmid. Method parameters are summarized in Table 2-1. Comparisons of the relative fluorescence of SC pDNA versus OC and L pDNA were made by analyzing equivalent amounts as described above and comparing the resulting fluorescence. It was found that on a weight to weight ratio, SC pDNA was only 59% as fluorescent relative to L pDNA by agarose gel analysis. To correct for this difference, SC pDNA amounts were multiplied by 1.7 prior to analysis. This difference has been reported previously and is likely due to the relative inaccessibility of ethidium bromide to the SC topology (Cantor 1980). Percent recovery using the phenol: chloroform: isoamyl alcohol method was found not to be dependent on topoform. Recovery was 90 (± 6) % for SC and 86 (± 13) % for L pDNA. Comparisons of the relative fluorescence of SC pDNA versus L pDNA were made by digesting SC pDNA with the Hind III restriction enzyme (Promega, Madison, WI) which has a single recognition site in the plasmid. Equivalent amounts of L and SC pDNA were then loaded on agarose gels as described above and the relative fluorescence compared. Percent recovery was calculated by comparing phenol: chloroform: isoamyl alcohol (25: 24: 1, v/v/v) extracted versus non-extracted known amounts and analyzing on agarose gels as described above.

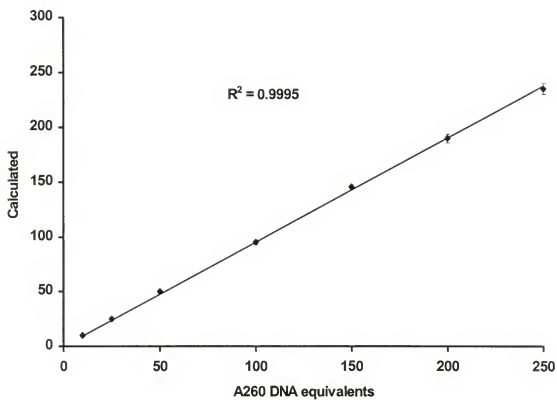


Figure 2-4. Standard curve for supercoiled pDNA. Abcissa represents total ng estimated by UV absorbance at 260 nm. Ordinate represents ng calculated by the KDS 1D software. Error bars represent ± 1 standard deviation.

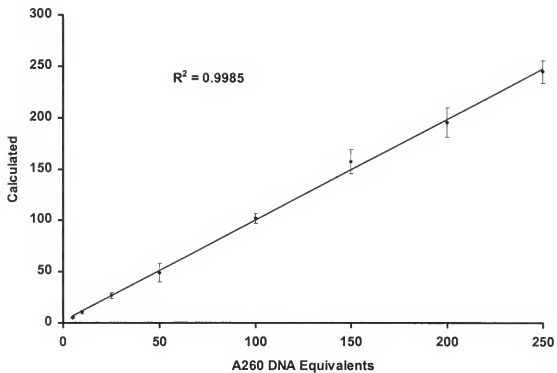


Figure 2-5. Standard curve for OC pDNA. Abcissa represents total ng estimated by UV absorbance at 260 nm. Ordinate represents ng calculated by the KDS 1D software. Error bars represent ± 1 standard deviation.

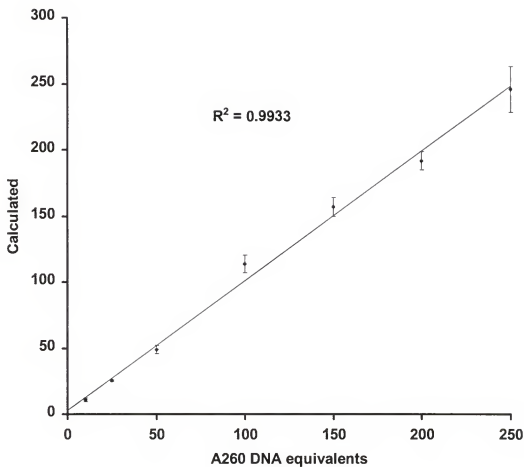


Figure 2-6. Standard curve for Linear pDNA. Abcissa represents total ng estimated by UV absorbance at 260 nm. Ordinate represents ng calculated by the KDS 1D software. Error bars represent ± 1 standard deviation.

Table 2-1. Method parameters for pDNA analysis.

Accuracy	Supercoiled: 94-101 % Open Circular: 98-108 % Linear: 96-113 %
Precision	Supercoiled: ≤ 5 % Open Circular: ≤ 19 % Linear: ≤ 13 %
Lower Limit of Quantitation	0.5 ng/ μ l
Lower Limit of Detection	0.25 ng/ μ l
Recovery from Plasma	Supercoiled: 90 (± 6) % Linear: 86 (± 13) %

Theoretical

The degradation of SC pDNA was assumed to follow pseudo first-order kinetics. The model used is diagrammed in Figure 2-7. In this model, pDNA degradation is considered to be a unidirectional process. The degradation of L pDNA is considered to yield fragments of heterogeneous lengths, thus these products were not included in the fitted model. No elimination from any of the compartments is assumed to occur through routes other than degradation to the following topoform.

Based on this model the following differential equations were derived to describe the process:

$$\frac{dSC}{dt} = -k_s \cdot SC$$

$$\frac{dOC}{dt} = k_s \cdot SC - k_o \cdot OC$$

$$\frac{dL}{dt} = k_o \cdot OC - k_l \cdot L$$

The amounts of supercoiled, open circular and linear pDNA were then fit to the integrated form of the equations:

$$SC = SC_0 \cdot e^{-k_s \cdot t}$$

$$OC = k_s \cdot SC_0 \cdot \left(\frac{1}{k_s - k_o} \cdot e^{-k_o \cdot t} + \frac{1}{k_o - k_s} \cdot e^{-k_s \cdot t} \right)$$

$$L = k_o \cdot k_s \cdot SC_0 \cdot \left(\frac{1}{(k_s - k_o)(k_o - k_l)} \cdot e^{-k_o \cdot t} + \frac{1}{(k_o - k_s)(k_s - k_l)} \cdot e^{-k_s \cdot t} + \frac{1}{(k_s - k_o)(k_s - k_l)} \cdot e^{-k_l \cdot t} \right)$$

Where SC, OC, and L are the amounts of supercoiled, open circular, and linear pDNA present at time=t, respectively. SC_0 is the amount of supercoiled pDNA present at time (t)=0. The constants k_o , k_s , and k_l represent the rate constants for the degradation of SC, OC, and L pDNA respectively. The constants represent the activity of all enzymes acting in the degradation process. Non-linear curve fitting and goodness of fit, model selection

criteria (MSC) assessment was carried out using Scientist (version 4.0, Micromath, Salt Lake City, UT) (MicroMath 1995). Area under the plasma concentration time curve (AUC) was calculated using trapezoidal rule. Area under the terminal portion of the plasma concentration time curve, AUC_{term} , was calculated by integration using the equation:

$$AUC_{term} = \frac{C_{last}}{k}$$

Where C_{last} is the last concentration point measured and k is the terminal elimination rate constant. Clearance (Cl) was calculated from the volume (V) of rat plasma (7.8 ml) and the terminal elimination rate constant (k) using the equation (Davies 1993):

$$Cl = V \cdot k$$

Statistical analysis was performed using SAS (The SAS Institute, Cary, NC).

Results

For quantitative purposes, the relative fluorescence of SC pDNA was compared to that of OC and L pDNA. It was found that on a weight to weight ratio, SC pDNA was only 59% as fluorescent relative to L pDNA by agarose gel analysis. To correct for this difference, SC pDNA amounts were multiplied by 1.7 prior to analysis. This difference has been reported previously, and is likely due to the relative inaccessibility of ethidium bromide to the SC topology (Cantor 1980). Percent recovery using the phenol: chloroform: isoamyl alcohol method was found not to be dependent on topoform. Recovery was 90 (± 6) % for SC and 86 (± 13) % for L pDNA.

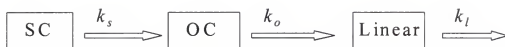


Figure 2-7. Pharmacokinetic model of plasmid DNA degradation in rat plasma. The model is considered to be a unidirectional process. SC, OC, and L represent the amounts of supercoiled, open circular, and linear plasmid, respectively, in each compartment. The rate constants k_s , k_o , and k_l represent the degradation constants for supercoiled, open circular, and linear plasmid, respectively.

Figure 2-8 displays a representative gel in which the degradation of SC pDNA and the appearance of OC and L topoforms of plasmid is observed. In addition, the degradation products of L pDNA are visible as a light smear running below the band at 60 min. Under the conditions used in this experiment, limit of quantification was 0.5 ng/ μ l using the Lambda Hind III size standard. Plasmid amounts were calculated from agarose gel analysis using Kodak Digital Science 1D image analysis software (Eastman Kodak, Rochester, NY) as described in the methods section. The observed and predicted values, based on the model displayed in Figure 2-8, are plotted in Figure 2-9. Plasmid concentrations were well described the model, MSC=3.0. Pharmacokinetic parameters calculated based on the model are summarized in Table 2-2. SC pDNA degraded rapidly in the plasma with a half-life of $1.2 (\pm 0.1)$ min. OC plasmid however was fairly stable, degrading with a half-life of $21 (\pm 1)$ min. L plasmid degraded more rapidly than the OC topoform but was fairly stable, in comparison to the SC plasmid degrading with a half-life of $11 (\pm 2)$ min. OC AUC was nearly 17 times larger than SC, and 2.3 times larger than L pDNA (Table 2-3).

No kinetics suggestive of enzyme saturation were observed under the experimental conditions tested. However, to ensure that saturation of plasma nucleases was not resulting in artificially low rate constant values, we analyzed the rate constants produced in dilute plasma (dilution was chosen as decreasing the dose of pDNA quickly results in a loss of sensitivity and sample sizes too large for loading). If saturation of

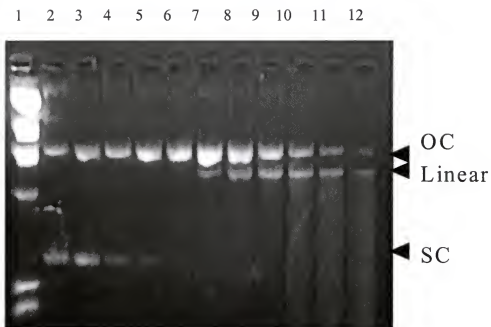


Figure 2-8. Agarose gel analysis of pDNA degradation in isolated rat plasma. Lane 1; size standard, lane 2; 30 sec, lane 3; 1 min, lane 4; 2 min, lane 5; 3 min, lane 6; 5 min, lane 7; 10 min, lane 8; 20 min, lane 9; 30 min, lane 10; 45 min, lane 11; 60 min, lane 12; 80 min.

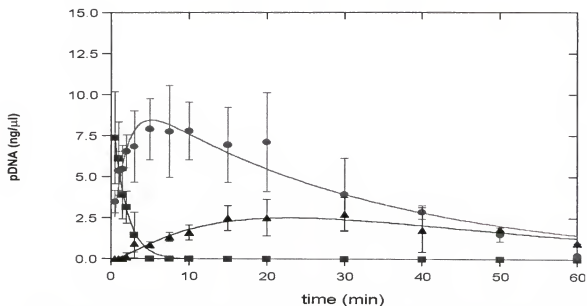


Figure 2-9. Experimental and fitted data based on the pharmacokinetic model described in the text. Data points represent actual experimental data. Lines represent values predicted by the model. Data represents mean of $n=3 \pm 1$ standard deviation. Key: ■ supercoiled, ● open circular, ▲ linear.

Table 2-2. Pharmacokinetic parameters for pDNA in isolated rat plasma

Topoform	Rate constant	Value (min^{-1})	Standard Deviation	Half-life (min)
Supercoiled	k_s	0.6	0.03	1.2 (± 0.1)
Open circular	k_o	0.03	0.002	21 (± 1)
Linear	k_l	0.06	0.008	11 (± 2)

Data represent the fitted values of $n=6 \pm 1$ standard deviation.

Table 2-3. Noncompartmental parameters for pDNA after incubation in isolated plasma.

Topoform	AUC (ng/μl*min)	Clearance (μl/min)
Supercoiled	18	360 ($\pm$ 9)
Open circular	310	23 ($\pm$ 1)
Linear	130	47 ($\pm$ 5)

Data represent $n=6 \pm$ standard deviation. AUC was calculated from the model fitted values using trapezoidal rule as described in the methods section. Clearance was calculated from the fitted rate constants and volume of rat plasma as described in the Methods section.

plasma nucleases was occurring, we expected that the rate constants in dilute plasma should deviate from a linear relationship negatively. Thus we tested the kinetics of pDNA degradation in 25% and 50% plasma. As displayed in Figure 2-10, no deviation was observed in the degradation of SC and OC pDNA.

To further investigate the mechanism responsible for the observed degradation, and further validate our assay (to ensure the assay was not causing degradation itself) we studied the degradation of pDNA in PBS diluted 25% heated plasma (90°C for 10 min) and PBS containing 0.1 mM EDTA. No degradation of SC pDNA was observed in either case through 1 hr (Figure 2-11). The degradation sensitivity to heat and EDTA provides evidence that the degradation observed in the assay is due to enzymatic processes.

We next sought to determine if the degradation observed in the previous experiments was dependent upon pDNA sequence. We, therefore, utilized the same model diagrammed above and replaced the pGL3 plasmid with the pGE150 plasmid. Unlike the pGL3 plasmid, which encodes for the luciferase protein and has an SV40 promoter, this plasmid encodes for the green fluorescent protein and includes a CMV promoter. If the degradation of pDNA in the plasma was sequence dependent, we expected the degradation rate constants observed to differ from those observed in the previous experiment. A comparison of plasma concentrations of OC and L pDNA is presented in Figures 2-12 and 2-13. The resulting rate constants are presented in Table 4.

Again the model diagrammed in Figure 2-7 described the data (model selection criteria = 3.1, $R^2=0.96$, 0.99, and 0.92 for SC, OC, and L respectively). To determine if

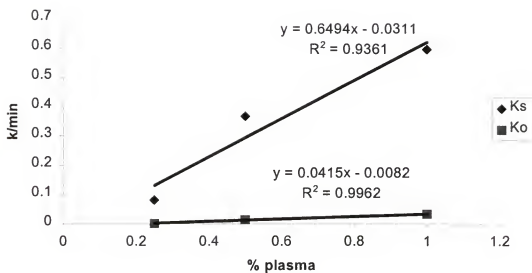


Figure 2-10. Analysis of rate constants in dilute plasma. Degradation rate constants were modeled in PBS diluted rat plasma. Rate constants represent the fitted values of $n=6$ rats/time point. Key: $\blacklozenge k_s$ in dilute plasma, $\blacksquare k_o$ in dilute plasma. The value of k_i is not reported due to the prolonged stability of linear plasmid in dilute plasma.

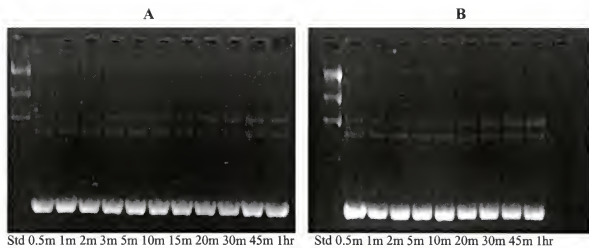


Figure 2-11. Degradation of supercoiled pDNA in 25% rat plasma after (A) incubating the plasma at 90°C for 10 min (B) the addition of 0.1 mM EDTA.

these rates were significantly different from those obtained using the pGL3 plasmid a statistical analysis was carried out using a 2-tailed equal variance student's t-test. The resulting parameters (k_o , and k_i) were not significantly different when judged at the $p < 0.05$ criteria. These results suggest that pDNA sequence is not a major factor involved in the overall degradation of pDNA by plasma nucleases.

Conclusions

Previous reports on the pharmacokinetics of pDNA have only been qualitative, or involved radiolabeling. These studies indicated that pDNA degrades within 5 minutes *in vitro* or after IV injection (Kawabata et al. 1995; Thierry et al. 1997). In this study, we sought to quantitatively model the pharmacokinetics underlying the stability of pDNA in the plasma. The results revealed that SC pDNA degrades in the plasma with a half-life of 1 min. OC pDNA is more stable than the SC topoform degrading with a half-life of 20 min. L pDNA is degraded more rapidly than the OC topoform. This latter shortened stability is likely due to the accessibility of various nucleases present in the plasma to the L pDNA. OC plasmid must be nicked by endonucleases on each sister strand in the same location to generate L pDNA. However L pDNA would be accessible to both endonucleases and exonucleases, thus degrading more rapidly. The model and equations presented successfully described the degradation of pDNA in the plasma.

Investigations by Thierry and coworkers suggested the main nuclease activity was endonucleolytic based on the finding that the L: SC ratio increased over time and the SC: OC ratio remained identical to control (Thierry et al. 1997). However this view fails to

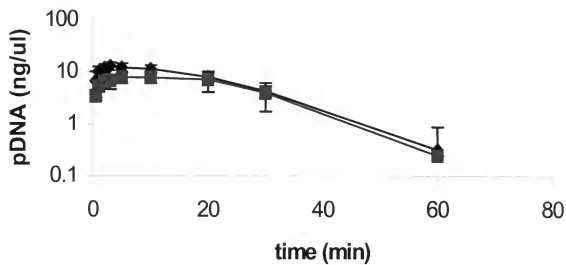


Figure 2-12. Comparison of concentrations of OC pDNA using (◆) pGE150 concentrations of OC pDNA using (■) pGL3 in isolated rat plasma. Data represents mean of $n=3 \pm 1$ standard deviation.

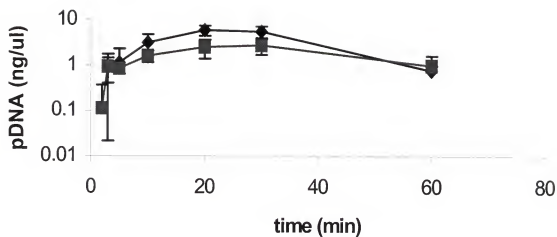


Figure 2-13. Comparison of concentrations of L pDNA using (◆) pGE150 versus concentrations of L pDNA using (■) pGL3 in isolated rat plasma. Data represents mean of $n=3 \pm 1$ standard deviation.

Table 2-4. Pharmacokinetic parameters for pDNA after incubating the pGE150 plasmid in isolated rat plasma.

Topoform	Rate constant	Value (min^{-1})	Standard Deviation	Half-life (min)
Open Circular	k_o	0.04	0.007	21 (± 1)
Linear	k_l	0.06	0.007	11 (± 1)

Data represent the fitted values of n=6 rats.

recognize endonucleolytic activity on L pDNA also generates degradation products. If endonuclease activity is the primary route of degradation, the kinetic ratios should all remain similar or decrease, owing to the fact that both exonucleases and endonucleases are active on the L pDNA and are thus both responsible for the observed degradation. Our results suggest that L pDNA has faster kinetics. This can be explained by endonuclease activity generating more free ends for degradation by exonucleases, exonucleases are more active than endonucleases, or that topoform influences the binding of these enzymes and thus influences reaction rate. Thus the main nuclease activity responsible for the observed kinetics remains to be answered.

Area under the curve analysis revealed that tissues would be exposed to the OC topoform predominantly after injection of naked pDNA (Table 2-2). Blood flow through any individual organ becomes the limiting factor in its ability to uptake a drug, which is highly metabolized in the plasma. When compared to hepatic plasma flow in the rat (8.14 ml/min), clearance values for the degradation of SC plasmid (4.6 ml/min) suggest that metabolism in the bloodstream is a major pathway by which *in vivo* clearance of SC pDNA can occur (Davies 1993). However, given that the clearance by degradation in the plasma is less than the liver blood flow, it also suggests that the liver possess a perfusion rate sufficient for uptake of SC pDNA after IV injection. This parallels the findings of Kawabata and coworkers who observed that naked plasmid was cleared more rapidly from the circulation after IV injection than after *in vitro* incubation in whole blood (Kawabata et al. 1995). Lung, kidney, and spleen have also been shown to take up detectable amounts of plasmid after IV injection (Kawabata et al. 1995; Osaka et al.

1996). Our model establishes not only that tissue uptake of plasmid is possible, but also that tissue uptake of the non-nicked SC topoform is possible after IV injection.

In summary this study presents a pharmacokinetic model describing the degradation of pDNA in rat plasma. A pharmacokinetic model is presented that can be of use in the future as gene therapy moves toward clinical trials. Using the derived model, we are able to conclude that naked SC pDNA degrades in rat plasma with a half-life of $1.2 (\pm 0.05)$ min, OC with a half-life of $21 (\pm 1)$ min, and L pDNA with a half-life of $11 (\pm 2)$ min.

CHAPTER 3

PHARMACOKINETICS OF PLASMID DNA AFTER IV BOLUS ADMINISTRATION IN THE RAT

Introduction

Naked pDNA is being used successfully in gene delivery by administration IM or SQ, (Haensler et al. 1999; Noll et al. 1999; Osorio et al. 1999; Rizzuto et al. 1999) and after IV injection (Wang et al. 1995; Budker et al. 1998; Song et al. 1998; Liu 1999; Zhang et al. 1999) in rats and mice. The success in these studies indicates that gene therapy is an attractive means by which to achieve therapeutic response. Thus, a thorough understanding of the pharmacokinetics of naked pDNA is an important area to be considered in order to move towards use in clinical trials

The pharmacokinetics of pDNA after IV bolus administration have been investigated using radiolabeling with linearized [^{33}P] pDNA (Osaka et al. 1996). These investigations have led to the conclusion that the half-life of the pDNA radiolabel is 7 to 12 min after IV bolus administration of naked pDNA in mice. However, this analysis offers no information on the other functional forms of the plasmid; supercoiled (SC), open circular (OC), or linear (L), nor does it discriminate the free label. Other studies have qualitatively revealed that the SC topoform of pDNA is not detectable as early as one minute post IV injection in mice (Lew et al. 1995; Thierry et al. 1997). The OC form of the plasmid has a half-life estimated in these studies to be in the range of 10 to 20 minutes (Thierry et al. 1997).

The purpose of this investigation was to model the pharmacokinetics of naked pDNA in a topoform specific manner after IV bolus administration in the rat. We further sought to determine if the observed pharmacokinetics were affected by changes in plasmid sequence. These results were then compared to pDNA degradation in isolated plasma in order to determine the relative importance of plasma nucleases in the pharmacokinetics of pDNA.

Methods

Animals (male Sprague-Dawley rats 300-350 g) were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed in the University of Florida Animal Resources Unit prior to all experiments and were given food and water ad libitum. Animals were anesthetized by intraperitoneal injection with 0.5 ml of a cocktail containing 13 mg/kg Xylazine, 2.15 mg/kg Acepromazine (The Butler Co., Columbus, OH), and 66 mg/kg Ketamine (Fort Dodge Animal Health, Fort Dodge Iowa).

Phenol: chloroform: isoamyl alcohol (25: 24: 1, v/v/v), chloroform, Tris, boric acid, EDTA, and agarose were purchased from Sigma Chemical Company (St. Louis, MO). Ethidium bromide (electrophoresis grade) was purchased from Fisher Biotech (Fair Lawn, NJ). Competent JM109 bacteria (Promega, Madison, WI) were transformed according to the manufacturer's directions with the pGL3 control plasmid (Promega, Madison, WI) or pGeneMax plasmid (Gene Therapy Systems, San Francisco, CA). Plasmid DNA was isolated from overnight cultures using alkaline lysis and ultracentrifugation with a CsCl gradient (Katz 1977). Isolated pDNA was resuspended in phosphate buffered saline. Plasmid was >90% SC by agarose gel analysis.

To facilitate blood sampling, male Sprague-Dawley rats (300-350g) were anesthetized and the jugular vein was exposed via an incision, isolated, ligated, and

nicked with ophthalmic scissors. A sterile silastic (0.640 cm internal diameter by 0.12 cm outer diameter, 10 cm in length) filled with sterile saline was threaded 30-40 mm into the jugular vein and positioned just distal to the entrance to the right atrium and secured by 6.0 silk sutures (Figure 3-1). For injections, the femoral vein was isolated, and pDNA was injected into the femoral vein using a 27-gauge needle (Figure 3-2). Isolated blood samples (approx. 300 μ l) were drawn through the jugular vein cannula and immediately placed in test tubes containing 0.57 ml of 0.34 M EDTA (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) on ice at the times indicated. This concentration of EDTA has previously been shown to inhibit the degradation of pDNA in isolated rat plasma (Houk 1999).

To isolate pDNA from whole blood samples, 250 μ l of blood was liquid/ liquid extracted with 250 μ l of phenol: chloroform: isoamyl alcohol (25: 24: 1, v/v/v), vortexed for 5 s at low speed, and centrifuged at 20,800 x g for 10 min at room temperature. The aqueous phase was removed and stored at -20°C until analysis.

Samples were loaded on 0.8% agarose in 0.9 M Tris-borate, 1 mM EDTA gels containing 0.001% ethidium bromide. Electrophoresis was carried out at 0.30 V/cm³ for 12h. The ethidium bromide was excited on a UV light box (Model TFX-35M, Life Technologies, Grand Island, NY) and the net fluorescence intensity captured at 590 nm on a Kodak DC120 digital camera (Eastman Kodak, Rochester, NY). The amounts of SC, OC, and L pDNA were calculated using Kodak Digital Science 1D Image Analysis Software (version 3.0, Eastman Kodak Company, Rochester NY) with a Lambda Hind III digest (Promega, Madison WI) and a High DNA Mass Ladder (Life Technologies, Grand



Figure 3-1. Photograph of the jugular cannula placement used for blood sampling.



Figure 3-2. Photograph of the femoral vein isolation and injection procedure used for IV bolus administration.

Island, NY) as external standards. Accuracy was 96 to 104% for SC pDNA, 98 to 108% for OC, and 96 to 113% for L pDNA. Percent coefficient of variation was $\leq 5\%$, 19%, and 13% for SC, OC and L forms of the plasmid respectively. Standard curves for all three forms of the plasmid (Figures 2-1, 2-2, and 2-3) were linear between 10 and 250 ng pDNA bands ($R^2 = 0.9995$, 0.9985, and 0.9933 for SC, OC, and L, respectively). All reported concentrations were calculated from bands within the range of the standard curves. Lower limit of quantitation was 0.5 ng/ μ l for all three forms of the plasmid. Lower limit of detection was 0.25 ng/ μ l for all three forms of the plasmid. Method parameters are summarized in Table 2-1. Comparisons of the relative fluorescence of SC pDNA versus OC and L pDNA were made by analyzing equivalent amounts as described above and comparing the resulting fluorescence. It was found that on a weight to weight ratio, SC pDNA was only 59% as fluorescent relative to L pDNA by agarose gel analysis. To correct for this difference, SC pDNA amounts were multiplied by 1.7 prior to analysis. This difference has been reported previously, and is likely due to the relative inaccessibility of ethidium bromide to the SC topology (Cantor 1980). Percent recovery using the phenol: chloroform: isoamyl alcohol method was found not to be dependent on topoform. Recovery was 90 (± 6) % for SC and 86 (± 13) % for L pDNA.

Theoretical

The degradation of SC pDNA was assumed to follow pseudo first-order kinetics. The model used is diagrammed in Figure 2-3. In this model, pDNA degradation is considered to be a unidirectional process. The degradation of L pDNA is considered to yield fragments of heterogeneous lengths, thus these products were not included in the

fitted model. No elimination from any of the compartments is assumed to occur through routes other than degradation to the following topoform.

Based on this model the following differential equations were derived to describe the process:

$$\begin{aligned}\frac{dSC}{dt} &= -k_s \cdot SC \\ \frac{dOC}{dt} &= k_s \cdot SC - k_o \cdot OC \\ \frac{dL}{dt} &= k_o \cdot OC - k_l \cdot L\end{aligned}$$

The amounts of supercoiled, open circular and linear pDNA were then fit to the integrated form of the equations:

$$\begin{aligned}SC &= SC_0 \cdot e^{-k_s \cdot t} \\ OC &= k_s \cdot SC_0 \cdot \left(\frac{1}{k_s - k_o} \cdot e^{-k_o \cdot t} + \frac{1}{k_o - k_s} \cdot e^{-k_s \cdot t} \right) \\ L &= k_o \cdot k_s \cdot SC_0 \cdot \left(\frac{1}{(k_s - k_o)(k_l - k_o)} \cdot e^{-k_o \cdot t} + \frac{1}{(k_o - k_s)(k_l - k_s)} \cdot e^{-k_s \cdot t} + \frac{1}{(k_o - k_s)(k_s - k_l)} \cdot e^{-k_l \cdot t} \right)\end{aligned}$$

Where SC, OC, and L are the amounts of supercoiled, open circular, and linear pDNA present at time=t, respectively. SC_0 is the amount of supercoiled pDNA present at time (t)=0. The constants k_s , k_o , and k_l represent the rate constants for the degradation of supercoiled, open circular, and linear pDNA, respectively. The constants represent the activity of all enzymes acting in the degradation process. Non-linear curve fitting and statistical analysis was carried out using Scientist (version 4.0, Micromath, Salt Lake

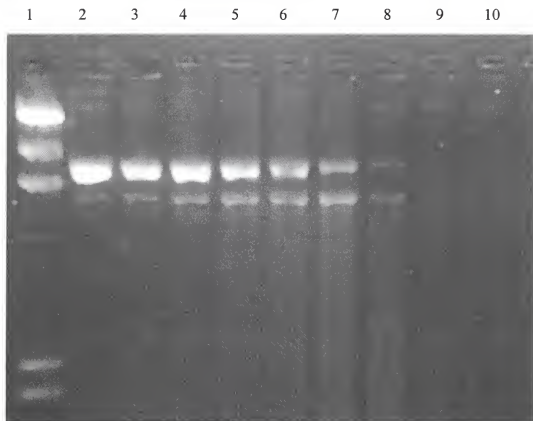


Figure 3-3. A representative gel from which plasmid amounts were quantified as described in the methods section. Lane 1: size standard, lane 2: 1 min, lane 3: 2 min, lane 4: 3.5 min, lane 5: 5 min, lane 6: 10 min, lane 7: 20 min, lane 8: 30 min, lane 9: 45 min, lane 10: 60 min.

City, UT). Noncompartmental pharmacokinetic analysis was carried out using standard parameters (Gibaldi 1982).

Results

SC pDNA was not detected as early as 30 seconds post-injection. The OC and L forms of the pDNA remained detectable through 30 minutes post-injection of the 500 μ g dose. An agarose gel analysis of the isolated samples is presented in Figure 3-3.

An important parameter to be considered is the initial concentrations achieved after IV administration in comparison to the initial concentrations *in vitro*. The initial concentrations of SC pDNA in the *in vitro* experiments (i.e. the time=0 concentration) were 10 ($\pm$ 0.3) ng/ μ l. After IV bolus administration, the initial extrapolated SC pDNA concentrations were 17 ($\pm$ 5) ng/ μ l. Thus, we concluded that these concentrations were within a range relevant for comparison. The observed and fitted concentrations of OC and L pDNA are presented in Figure 3-4. The model again adequately described the data, model selection criteria=4.42. Pharmacokinetic parameters calculated based upon the model are presented in Table 3-1.

A comparison of the *in vitro* and *in vivo* concentrations of OC and L pDNA are presented in Figures 3-5 and 3-6, respectively. Calculated pharmacokinetic parameters are presented in Table 3-2. OC pDNA half-life was markedly shorter after IV bolus administration than after incubation in isolated plasma, 5.3 ($\pm$ 1.4) versus 21 ($\pm$ 1) min. L pDNA removal was also more rapid after IV bolus administration, 1.9 ($\pm$ 0.8) versus 11 ($\pm$ 2) min after incubation in isolated plasma.

In order to further investigate the importance of plasmid sequence on the observed pharmacokinetics, we injected the pGeneMax-Luciferase, and pGE150 plasmids by IV

bolus administration at equivalent dose (500 µg). Concentrations of OC and L pDNA in the bloodstream are presented in Figures 3-7 and 3-8 respectively. The fitted elimination rate constants for OC and L pDNA were compared by 2-way ANOVA. The results are displayed in Table 3-3. There were no significant differences between the terminal rate constants of any of the 3 plasmids by 2-way ANOVA when judged at the $p < 0.05$ criteria.

Conclusions

DNase I is a well characterized enzyme in human plasma present at concentrations averaging 26.1 (± 9.2) ng/ml in the sera of normal humans (Chitrabamrung 1981). Traditionally, the presence of this enzyme has led to the conclusion that pDNA administered IV is degraded rapidly (Gosse et al. 1965; Chused 1972). This has led to the current view of gene delivery, where protection from plasma nucleases is a major goal of delivery systems. The results of this study demonstrate that although the half-life of SC and OC pDNA is remarkably short, degradation alone was not enough to explain the rapid disappearance of pDNA from the circulation observed *in vivo*. After IV bolus the rate of degradation of SC pDNA was greater than 7 times faster than in isolated plasma (Houk 1999).

Chused and coworkers (Chused 1972) also suggested that nuclease activity was not enough to explain the rapid clearance of KB cell DNA from the circulation in mice. In this study, only 2 to 3 % of the radioactivity was hydrolyzed to trichloroacetic acid (TCA) soluble fragments in 30 min, which was several half-lives longer than in the circulation. Tsumita and Iwanga (Tsumita and Iwanga 1963) also found that less than 5 % of the total radioactivity was found in the TCA soluble fraction after 4.5 hours in mouse serum.

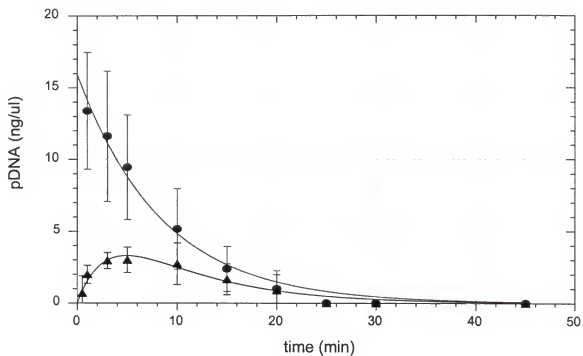


Figure 3-4. Experimental and fitted data based on the pharmacokinetic model described in the text. Data points represent actual experimental data. Lines represent values predicted by the model. Data represents mean of $n=6 \pm 1$ standard deviation. Key: ● open circular, ▲ linear.

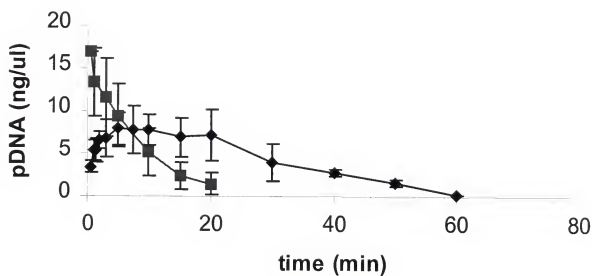


Figure 3-5. Concentrations of OC pGL3 after ■: IV bolus administration of a 500 μ g dose of SC pGL3, and ◆: Incubation of SC pGL3 in isolated plasma at 37°C.

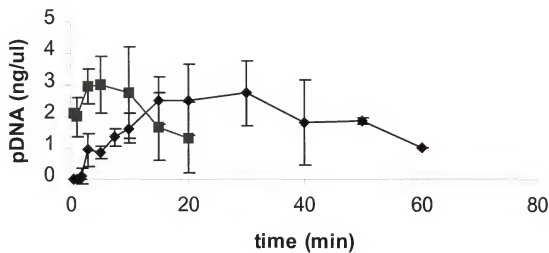


Figure 3-6. Concentrations of L pGL3 after ■: IV bolus administration of a 500 μ g dose of SC pGL3, and ◆: Incubation of SC pGL3 in isolated plasma at 37°C.

Table 3-1. Pharmacokinetic parameters calculated after 500 μg dose of SC pDNA.

Topoform	Rate constant	Value (min^{-1})	Standard Deviation	Half-life (min)
Supercoiled	k_s	3.4	0.4	0.2 (± 0.03)
Open circular	k_o	0.14	0.04	5.3 (± 1.4)
Linear	k_l	0.41	0.18	1.9 (± 0.8)

Parameters represent averages of $n=6$ rats.

Table 3-2. Comparison of *in vivo* and *in vitro* pharmacokinetic parameters for pDNA.

Topoform	Terminal Half-life (min)		AUC ∞ (ng/ μ l*min)		Cl/f (μ l/min)	
	<i>In vitro</i>	<i>In vivo</i>	<i>In vitro</i>	<i>In vivo</i>	<i>In vitro</i>	<i>In vivo</i>
Supercoiled	1.2 (± 0.1)	?	17(± 5)	N/A	360 (± 9)	N/A
Open Circular	21 (± 1)	5.3 (± 1.4)	280 (± 150)	128 (± 52)	23 (± 1)	4800 (± 2000)
Linear	11(± 2)	1.9 (± 0.8)	103 (± 47)	49 (± 28)	47 (± 5)	11000 (± 5000)

Parameters represent averages of n=3 (± 1 standard deviation).

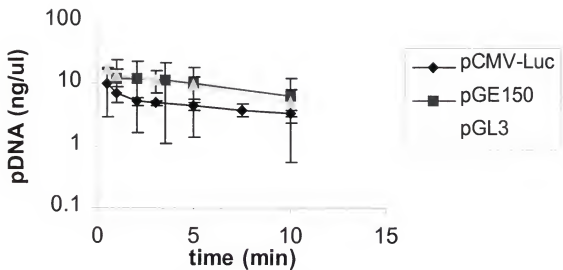


Figure 3-7. Concentrations of OC pDNA in the bloodstream after IV bolus administration of 500 μ g of pCMV-Luc, pGE150, or pGL3. Data represents mean of $n=3 \pm 1$ standard deviation.

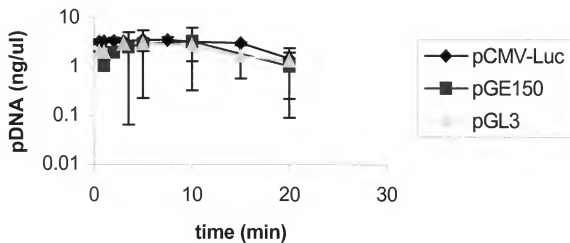


Figure 3-8. Concentrations of L pDNA in the bloodstream after IV bolus administration of 500 μ g of pCMV-Luc, pGE150, or pGL3. Data represents mean of $n=3 \pm 1$ standard deviation.

Table 3-3. Comparison of OC and L pDNA parameters after administration of SC pGL3, pGE150, and pGeneMax.

Plasmid	Parameter	OC Value	L Value
pGL3	AUC_{∞} (ng/ μ l*min)	120 ($\pm$ 50)	52 ($\pm$ 25)
	C_{max} (ng/ μ l)	13 ($\pm$ 4)	3.2 ($\pm$ 1.0)
pGE150	AUC_{∞} (ng/ μ l*min)	160 ($\pm$ 30)	55 ($\pm$ 12)
	C_{max} (ng/ μ l)	14 ($\pm$ 3)	3.3 ($\pm$ 0.9)
pGeneMax	AUC_{∞} (ng/ μ l*min)	121 ($\pm$ 25)	59 ($\pm$ 3)
	C_{max} (ng/ μ l)	12 ($\pm$ 5)	3.7 ($\pm$ 0.3)

Parameters represent averages of n=3 ($\pm$ 1 standard deviation).

Alternatively, Gosse and coworkers suggested a major role for nucleases in the initial degradation of DNA after IV administration in rabbits and mice (Gosse et al. 1965). This finding was based upon the proportionality between the initial rate of depolymerization and the plasma DNase activity level. Also, a rapid decrease in viscosity of isolated blood was discovered indicating a depolymerization of DNA. Finally, a markedly slower disappearance of DNA-methyl green complex (a non-specific DNase inhibitor) than after native DNA.

The reason for this disparity in results deserves further investigation. Gosse utilized much higher doses of pDNA than Chused and coworkers in their investigations, 200 μg versus 5 μg pDNA in Chused and coworkers' investigations. This disparity may be due to saturation of a scavenger receptor, allowing nuclease activity to become increasingly important. The effect of increasing dose on the clearance of DNA deserves further investigation.

The results presented in the present study indicate that SC pDNA was undetectable after IV bolus administration, whereas SC pDNA was readily detectable in isolated plasma, and remained detectable through 3 min of incubation. Similar results were seen for the OC and L forms of the plasmid. The half-lives of OC and L pDNA decreased from 21 (± 1) to 5.3 (± 1.4) and 11 (± 2) to 1.9 (± 0.8) min, respectively. Thus indicating that nuclease activity alone is not sufficient to describe the rapid clearance of pDNA from the bloodstream in rats. The observed kinetics were found not to be dependent upon plasmid sequence.

CHAPTER 4 DOSE DEPENDENCY OF PLASMID DNA PHARMACOKINETICS

Introduction

The studies presented in Chapters 2 and 3 have shown that degradation in the plasma alone was not sufficient to describe the pharmacokinetics of pDNA. After IV bolus administration SC pDNA was undetectable as early as 30 sec. This was in contrast to isolated plasma when SC pDNA was detectable 3 minutes after the start of incubation in isolated plasma. OC and L pDNA terminal half-life also decreased from 21 (± 1) to 5.3 (± 1.4) and 11 (± 2) to 1.9 (± 0.8) min, respectively.

Other investigators have suggested variable importance of plasma nucleases in the degradation of genomic DNA after IV bolus administration. For example, Chused and coworkers (Chused 1972), Whaley and Webb (Whaley 1972), and Tsumita and Iwanaga (Tsumita 1963) all suggested a minimal role for plasma nucleases in the clearance of DNA. This was based upon the observed fragmentation rate of genomic DNA in isolated plasma versus the fragmentation rate after IV bolus administration, and the diffuse high level of distribution of the DNA to tissues immediately after administration. This finding was accompanied by the suggestion of extensive uptake of intact DNA molecules by the reticuloendothelial system.

Alternatively, Gosse and coworkers (Gosse et al. 1965) found that "the plasma DNases play a fundamental and probably exclusive role in the initial degradation of DNA". This was based upon 3 observations. First was a rapid decrease in viscosity of

the blood within 3 minutes after administration. Second, this was based upon the proportionality between the initial rate of degradation and the DNase activity level. Third, this was also based upon the markedly slower disappearance of the DNA-methyl green complex (a non-specific DNase inhibitor).

The disparity between the results presented here and the previous studies deserves further investigation. Chused and coworkers, and Whaley and Webb, utilized smaller doses of DNA in their experiments versus Gosse and coworkers (5 versus 200 μg /mouse). If this large dose had temporarily saturated an alternative clearance mechanism, this would increase the observed importance of nucleases. Thus, nonlinear processes may provide an explanation for the observed disparity.

Nonlinear clearance of pDNA has previously been suggested using pharmacokinetic analysis of outflow patterns from rat perfused liver studies with radiolabeled OC pDNA (Yoshida 1996). In this study, V_d increased and extraction ratio decreased as perfusion dose was increased from 1.33 to 13.3 $\mu\text{g}/\text{liver}$.

The purpose of this investigation was to model the pharmacokinetics of increasing doses of naked pDNA in a topoform specific manner after IV bolus administration in the rat. This information may provide an explanation for the disparity between the results presented here and in previous studies. Furthermore, we sought to determine the metabolite (OC and L) pharmacokinetics independently, by direct injection of each of the metabolites. This information will provide a basis upon the percent conversion of the SC to the OC form and the OC form to the L form of the plasmid.

Methods

Animals (male Sprague-Dawley rats 300-350 g) were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed in the University of Florida Animal Resources Unit prior to all experiments and were given food and water ad libitum. Animals were anesthetized by intraperitoneal injection with 0.5 ml of a cocktail containing 13 mg/kg Xylazine, 2.15 mg/kg Acepromazine (The Butler Co., Columbus, OH), and 66 mg/kg Ketamine (Fort Dodge Animal Health, Fort Dodge Iowa).

Phenol: chloroform: isoamyl alcohol (25: 24: 1, v/v/v), chloroform, Tris, boric acid, EDTA, and agarose were purchased from Sigma Chemical Company (St. Louis, MO). Ethidium bromide (electrophoresis grade) was purchased from Fisher Biotech (Fair Lawn, NJ). Competent JM109 bacteria (Promega, Madison, WI) were transformed according to the manufacturers directions with the pGL3 control plasmid (Promega, Madison, WI) or pGeneMax plasmid (Gene Therapy Systems, San Francisco, CA). Plasmid DNA was isolated from overnight cultures using alkaline lysis and ultracentrifugation with a CsCl gradient (Katz 1977). Isolated pDNA was resuspended in phosphate buffered saline. Plasmid was >90% SC by agarose gel analysis.

OC pDNA was produced by incubation of the SC pDNA, in phosphate buffered saline, at 70°C for 16h. This procedure resulted in >90% OC plasmid (Figure 4-1). UV absorbance at 260 nm and the A260/A280 ratio of the pDNA solution did not change after this treatment (Figure 4-2).

L pDNA was produced by digestion with BamHI restriction enzyme (Promega, Madison, WI) in separate reaction mixtures containing 173 µl of DI H₂O, 27 µl 10x Buffer (Promega, Madison, WI) 56 µl (100 µg) pGL3, and 10 µl of BamHI (10 U/µl).

The reaction mixture was incubated at 37°C for 3 h. Plasmid was then isolated from the reaction mixture by extraction with 1 volume of phenol: chloroform: isoamyl alcohol (25: 24: 1), followed by extraction with 1 volume of chloroform. Plasmid was then concentrated by precipitation with 0.3 M Na Acetate, and 1 volume of isopropanol, followed by centrifugation at 13K g for 30 min at 4°C, and resuspended in 50 µl of phosphate buffered saline. This method routinely produced >90% L pDNA (Figure 4-3). Concentration of pDNA was measured by monitoring UV absorbance at 260 nm, purity was measured by A260/A280 ratio. A resulting purity of less than 1.7 was re-extracted with 1 volume of chloroform until purity >1.7 was achieved.

For blood sampling, male Sprague-Dawley rats (300-350g) were anesthetized and the jugular vein was exposed via an incision, isolated, ligated, and nicked with ophthalmic scissors. A sterile silastic (0.64 cm internal diameter by 0.12 cm outer diameter, 10 cm in length) filled with sterile saline was threaded 30-40 mm into the jugular vein and positioned just distal to the entrance to the right atrium and secured by 6.0 silk sutures. For injections, the femoral vein was isolated, and pDNA was injected into the femoral vein using a 27-gauge needle. This method is graphically illustrated in Figure 3-1 and 3-2. Isolated blood samples (approx. 300 µl) were drawn through the jugular vein cannula and immediately placed in test tubes containing 0.57 ml of 0.34 M EDTA (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) on ice at the times indicated. This concentration of EDTA has previously been shown to inhibit the degradation of pDNA in isolated rat plasma (Houk 1999).

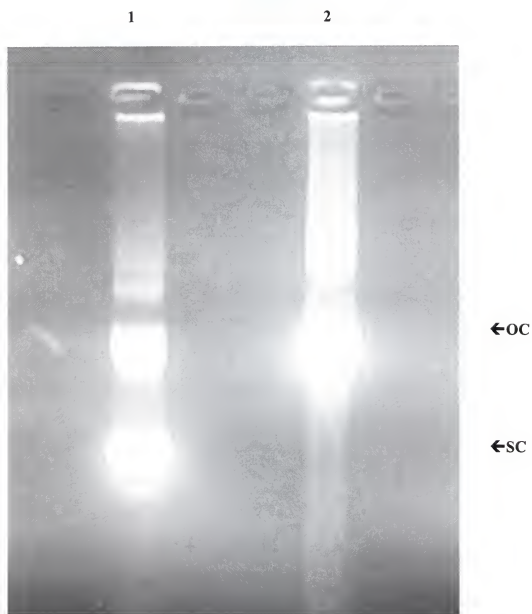


Figure 4-1 Agarose gel analysis of pDNA after conversion to the OC form of the plasmid. Lane 1: Prior to treatment plasmid is predominately SC. Lane 2: After treatment plasmid is completely converted to to OC form..

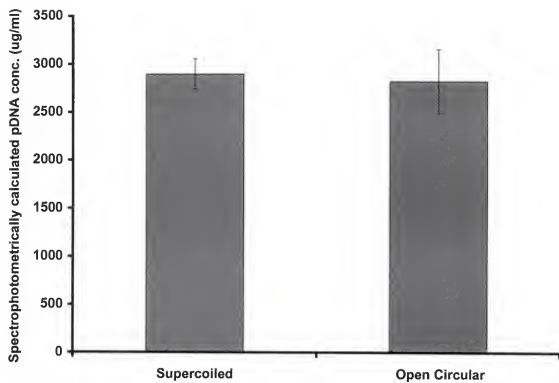


Figure 4-2. Absorbance of pDNA before and after conversion to the OC form. Data represents averages of $n=3 \pm 1$ standard deviation.

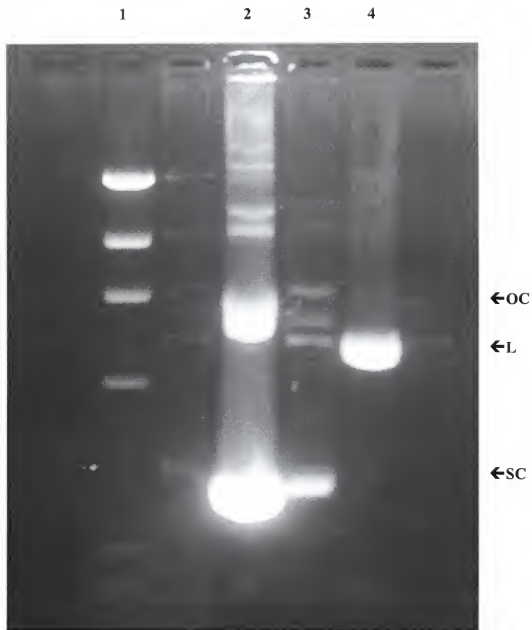


Figure 4-3. Agarose gel analysis of pDNA before and after conversion to the L form of the plasmid. Lane 1: Size standard, Lane 2: before treatment the plasmid is predominately in the SC and OC form, Lane 3: Mixture of SC, OC, and L plasmid for reference, Lane 4: after treatment the plasmid is completely converted to the L form.

To isolate pDNA from whole blood 250 μ l of blood was liquid/ liquid extracted with 250 μ l of phenol: chloroform: isoamyl alcohol (25: 24: 1, v/v/v), vortexed for 5 s at low speed, and centrifuged at 20,800 g for 10 min at room temperature. The aqueous phase was removed and stored at -20°C until analysis. Samples analyzed and quantitated as described in Chapter 2.

Results

SC pDNA was detectable in the bloodstream only after a 2500 μ g dose, no SC pDNA was detectable in the bloodstream at lower doses as early as 30 sec after administration. Because of this, the pharmacokinetic parameters reported for this form of pDNA relied only on data acquired from this dose. SC pDNA remained detectable in the plasma through 1 min after administration. Using the limited available data we approximated that SC pDNA degraded with a half-life of 0.15 ($\pm$ 0.01) min. The degradation of SC pDNA was fit to a one-compartment body model with central elimination and uptake (Figure 4-4). We extrapolated a least squares fit of the data to an initial, $t=0$, concentration which was necessary as this area accounted for a major portion of the AUC_{∞} . Clearance of SC pDNA was calculated to be 390 ($\pm$ 10) ml/min, and volume of distribution was 148 ($\pm$ 26) ml. (Table 4-1).

Concentrations of OC and L pDNA in the bloodstream after IV bolus administration are displayed in Figure 4-5 and 4-6 respectively. Noncompartmental analysis of all four doses is displayed in Table 4-2 for OC and Table 4-3 for L pDNA. A decrease in terminal slope is observable with increasing dose for the OC form of the plasmid (Figure 4-5). Clearance of the OC form of the plasmid decreased with increasing dose (Table 4-2). Formation clearance values for the OC form of the plasmid after the

administration of SC pDNA ranged from $1.3 (\pm 0.2)$ to $8.3 (\pm 0.8)$ ml/min for the 2500, and 250 μ g doses respectively. Formation clearance of the L form of the pDNA remained constant at an average of $6.7 (\pm 0.2)$ ml/min for all doses. The 250 μ g dose L concentrations close to limits of quantitation, and thus required a large amount of extrapolation for AUC calculation. For this reason, the 250 μ g dose L analysis was excluded from the noncompartmental analysis. Corresponding plots of OC pDNA plasma concentrations, normalized for dose, were not superimposable (Figure 4-7) (Gibaldi 1982).

To investigate the percent of SC plasmid that becomes OC as well as the percent OC plasmid that becomes L, we compared the AUC obtained after IV bolus administration of the OC and L forms of plasmid independently at 2500 and 250 μ g doses. Plasma concentrations of OC pDNA obtained after administration of 2500 and 250 μ g doses are displayed in Figure 4-8 and 4-9 respectively. Noncompartmental analysis of the OC form of the plasmid at each dose is displayed in Table 4-4. Clearance again decreased between the 250 and 2500 μ g doses $8.8 (\pm 2.4)$ to $1.3 (\pm 0.2)$ ml/min. Clearance also remained consistent with that observed after administration of SC pDNA at each dose, $8.8 (\pm 2.4)$ versus $8.3 (\pm 0.8)$ ml/min at the 250 μ g dose, and $1.3 (\pm 0.2)$ versus $1.3 (\pm 0.2)$ at the 2500 μ g dose. Volume of distribution of the OC form was 43 (± 15) ml.

Concentrations of L pDNA after administration of 2500 and 250 μ g doses of L pDNA are presented in Figure 4-10 and 4-11 respectively. Noncompartmental analysis is

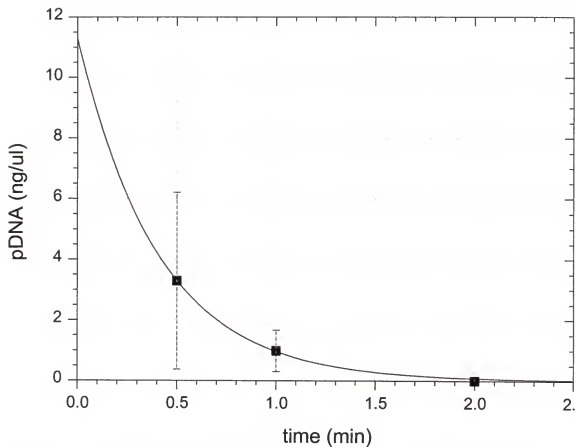


Figure 4-4. Concentrations of SC pDNA in the bloodstream after 2500 μg dose. SC pDNA remained detectable through 1 minute after administration. Data points represent averages of $n=3 \pm 1$ standard deviation. Lines represent a least squares fit of the data using the model described in the Methods section.

Table 4-1. Pharmacokinetic parameters estimated for supercoiled pDNA based upon the fit $t=0$ concentration of SC pDNA

Parameter	Value
AUC (ng/ μ l*min)	6.4 (± 0.2)
MRT(min)	0.21 (± 0.02)
Cl (ml/min)	390 (± 10)
Vdss (ml)	148 (± 26)
Half-life (min)	0.15 (± 0.02)

Parameters represent averages of $n=3 \pm 1$ standard deviation.

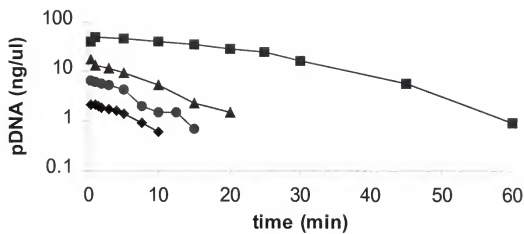


Figure 4-5. Concentrations of OC pDNA after IV bolus administration of: ■ 2500 µg, ▲ 500 µg, ● 333 µg, or ◆ 250 µg of SC pDNA. Data represents mean of n=3.

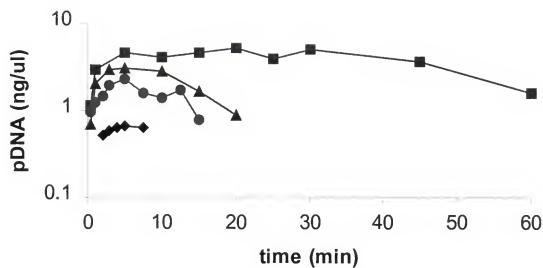


Figure 4-6. Concentrations of L pDNA after IV bolus administration of: ■ 2500 µg, ▲ 500 µg, ● 333 µg, or ♦ 250 µg of SC pDNA. Data represents mean of n=3.

Table 4-2. Noncompartmental analysis of OC pDNA after IV bolus administration of SC pDNA.

Parameter	2500 µg Dose	500 µg Dose	333 µg Dose	250 µg Dose
AUC (ng/µl*min)	1200 (±200)	120 (±50)	59 (±3)	18 (±2)
AUC % extrapolated	1 (±0.4)	9 (±4)	10 (±6)	24 (±2)
AUMC (ng/µl*min ²)	20000 (±6000)	1900 (±1200)	400 (±20)	130 (±20)
MRT (min)	16 (±3)	14 (±3)	6.8 (±0.4)	7.2 (±0.3)
Cl/f (ml/min)	2.1 (±0.4)	4.8 (±2.0)	5.7 (±0.3)	14 (±1)
Cl (ml/min)	1.3 (±0.2)	3.0 (±1.2)	3.5 (±0.2)	8.3 (±0.8)
C _{max} (ng/µl)	49 (± 4)	13 (±4)	6.5 (±0.3)	2.2 (±0.2)
t _{max} (min)	1	1	0.7 (± 0.3)	0.8 (± 0.3)

Parameters represent averages of n=3 (± 1 standard deviation).

Table 4-3. Noncompartmental analysis of L pDNA after IV bolus administration of SC pDNA.

Parameter	2500 µg Dose	500 µg Dose	333 µg Dose
AUC (ng/µl*min)	240 (±40)	52 (±25)	32 (±5)
AUC % extrapolated	12 (±7)	15 (±5)	13 (±7)
AUMC (ng/µl*min ²)	7500 (±2700)	570 (±370)	300 (±20)
MRT (min)	31 (±6)	10 (±2)	9.6 (±1.7)
Cl/f (ml/min)	10.6 (±2.0)	11 (±5)	11 (±1)
Cl (ml/min)	6.5 (±1.2)	6.9 (±2.8)	6.6 (±0.9)
C _{max} (ng/µl)	5.4 (± 0.6)	3.2 (±1.0)	2.4 (±0.5)
t _{max} (min)	22 (± 3)	5.3 (± 4.0)	6.0 (± 3.6)

Parameters represent averages of n=3 (± 1 standard deviation).

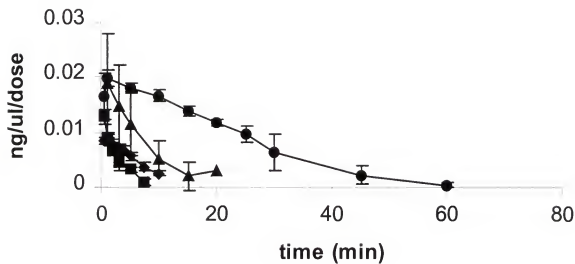


Figure 4-7. Superposition of OC pDNA concentrations normalized for dose after administration of ●: 2500 µg, ▲: 500 µg, ◆: 333 µg, or ■: 250 µg dose. Data represents mean of $n=3 \pm 1$ standard deviation.

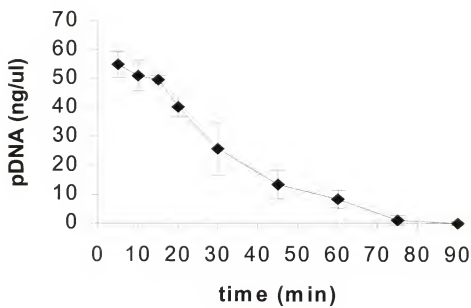


Figure 4-8. Concentrations of OC pDNA in the bloodstream after administration of OC pDNA at a 2500 μg dose. Data represents mean of $n=3 \pm 1$ standard deviation.

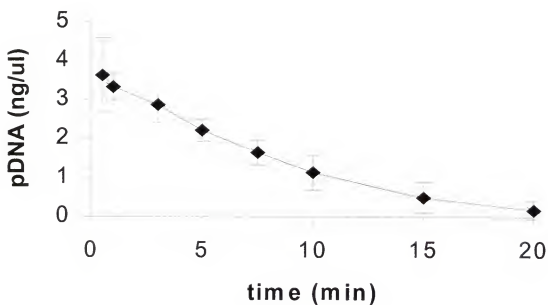


Figure 4-9. Concentrations of OC pDNA in the bloodstream after administration of OC pDNA at a 250 μg dose. Data represents mean of $n=3 \pm 1$ standard deviation.

Table 4-4. Noncompartmental analysis of OC pDNA after IV bolus administration of OC pDNA at 2500 and 250 μg doses.

Parameter	2500 μg	250 μg
AUC ($\text{ng}/\mu\text{l} \cdot \text{min}$)	1900 (± 200)	30 (± 9)
AUC % extrapolated	< 1	15 (± 2)
AUMC ($\text{ng}/\mu\text{l} \cdot \text{min}^2$)	50000 (± 5000)	220 (± 140)
MRT (min)	22 (± 1)	6.8 (± 2.3)
Cl (ml/min)	1.3 (± 0.2)	8.8 (± 2.4)
V _{d_{ss}} (ml)	29 (± 3)	56 (± 5)

Parameters represent averages of $n=3 \pm 1$ standard deviation.

presented in Table 4-5. The data was consistent with the clearance values observed after administration of SC pDNA, $7.6 (\pm 2.4)$ versus $6.6 (\pm 1.6)$. Volume of distribution for the L form of the plasmid was $38 (\pm 12)$ ml.

As is displayed in Figures 4-12 and 4-13, OC AUC after administration of SC pDNA was only $64 (\pm 11)\%$ and $59 (\pm 11)\%$ of the AUC after administration of OC pDNA for the 2500 and 250 μg doses respectively. The AUC of L pDNA after administration of OC pDNA was $105 (\pm 23)$ and $95\% (\pm 11)$ of the AUC of the AUC after administration of L pDNA for the 2500 and 250 μg doses respectively (Figures 4-14 and 4-15 respectively).

Conclusions

The results of this study reveal that all forms of pDNA (SC, OC, and L) are rapidly cleared from the circulation. Other investigators have qualitatively commented on the rapid clearance observed after IV bolus administration of pDNA (Lew et al. 1995; Mahato et al. 1995; Thierry et al. 1997). However, these studies have been limited to the OC and L forms of the plasmid. The half-life of the SC topoform has been unable to be estimated due to lack of detection (Lew et al. 1995; Thierry et al. 1997). Thierry and coworkers (Thierry et al. 1997) utilized electrophoresis and estimated the half-life of the OC form of the plasmid to be in the range of 10 to 20 min at a dose of 3.5 $\mu\text{g/g}$ in mice. This corresponds to a dose of approximately 1100 μg in a rat and is in reasonable agreement with the terminal half-lives that we observed here between the 500 and 2500 μg doses. Osaka and coworkers (Osaka et al. 1996) utilized a dose of 2.25 $\mu\text{g/g}$ of linearized radiolabeled plasmid and found the half-life to be 6.6 and 11.5 min ($n=2$). This corresponds to an approximate dose of 730 μg in a rat. We found the half-life of

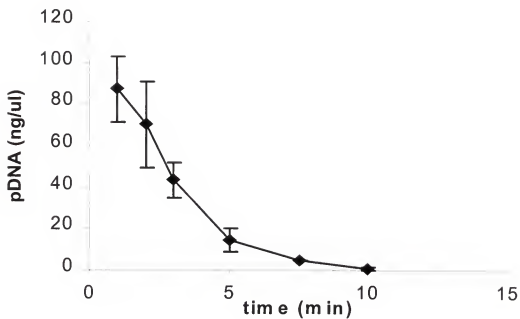


Figure 4-10. Concentrations of L pDNA in the bloodstream after administration of L pDNA at a 2500 μg dose. Data represents averages of $n=3 \pm 1$ standard deviation.

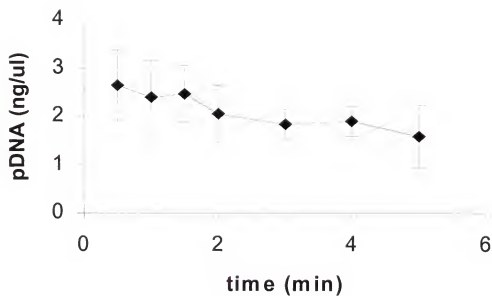


Figure 4-11. Concentrations of L pDNA in the bloodstream after administration of L pDNA at a 250 µg dose. Data represents averages of $n=3 \pm 1$ standard deviation

Table 4-5. Noncompartmental analysis of L pDNA after IV bolus administration of L pDNA at 2500 and 250 μg doses

Parameter	2500 μg	250 μg
AUC ($\text{ng}/\mu\text{l} \cdot \text{min}$)	330 (± 40)	22 (± 4)
AUC % extrapolated	3.0 (± 1.0)	51 (± 16)
AUMC ($\text{ng}/\mu\text{l} \cdot \text{min}^2$)	1500 (± 200)	77 (± 24)
MRT (min)	4.5 (± 0.1)	3.5 (± 1.2)
Cl (ml/min)	7.6 (± 0.8)	11 (± 2)
$V_{d_{ss}}$ (ml)	34 (± 4)	51 (± 17)

Parameters represent averages of $n=3 \pm 1$ standard deviation.

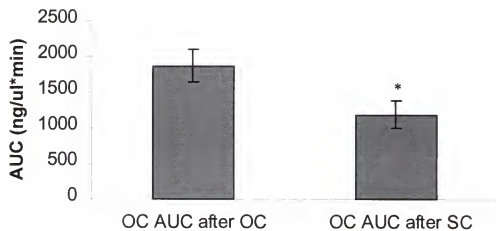


Figure 4-12. Area under the curve of OC pDNA after administration of a 2500 μ g dose of SC or OC pDNA. Data represents mean of $n=3 \pm 1$ standard deviation. * Indicates statistical significance by one way ANOVA ($p<0.05$).

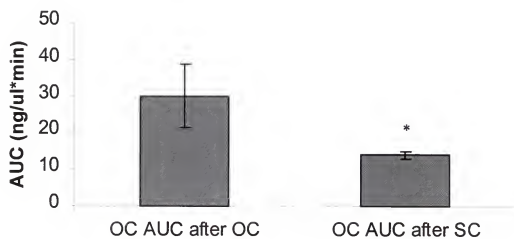


Figure 4-13. Area under the curve of OC pDNA after administration of a 250 µg dose of SC or OC pDNA. Data represents mean of $n=3 \pm 1$ standard deviation. * Indicates statistical significance by one way ANOVA ($p<0.05$).

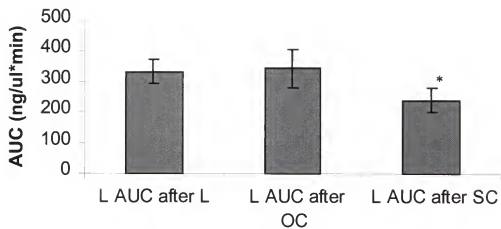


Figure 4-14. Area under the curve of L pDNA after administration of a 2500 µg dose of SC or OC pDNA. Data represents mean of $n=3 \pm 1$ standard deviation. * Indicates statistical significance by one way ANOVA ($p<0.05$).

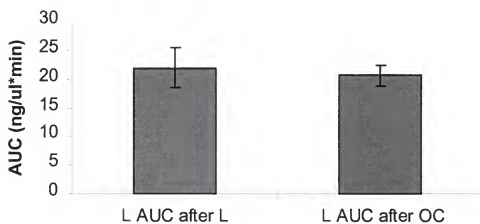


Figure 4-15. Area under the curve of L pDNA after administration of a 250 μg dose of SC or OC pDNA. Data represents mean of $n=3 \pm 1$ standard deviation. AUC differences were not statistically significant by one way ANOVA.

linear pDNA to be $1.7 (\pm 0.4)$ in our experiments. This difference may be due to species variation or an inability to differentiate the free radiolabel.

The SC form of the plasmid disappeared rapidly from the circulation. One possible explanation for the rapid disappearance of SC pDNA is that it is rapidly being converted to the OC form *in vivo* by endogenous plasma nucleases. Traditionally the presence of DNase I, which is present at concentrations averaging $26.1 (\pm 9.2)$ ng/ml in the sera of normal humans (Chitrabamrung 1981), has led to the conclusion that pDNA administered IV is degraded rapidly [Gosse, 1965 #20; Chused, 1972 #19]. If we compare the reported concentrations of DNase I in human plasma along with the reported SC pDNA nicking activity of DNase I under optimal conditions (Dwyer 1999), and make the assumption that the activity of rat DNase I is approximately the same as the human isoform (Takeshita et al. 1996), we can arrive at an approximate activity of 0.1 (ng pDNA/ μ l/min). This is far less than the *in vivo* SC pDNA nicking rate of 9.2 (ng pDNA/ μ l/min) observed 30 sec after administration of the 2500 μ g dose. One minute after administration the *in vivo* rate was 2.7 ng pDNA/ μ l/min. Thus, the activity of DNase I would not seem to be enough to describe the rapid conversion of SC pDNA to the OC form. The combined effect of enzymes in addition to DNase I is also insufficient to describe the kinetics in total. After IV bolus administration, the rate of degradation of SC pDNA was greater than 7 times faster than in isolated rat plasma.

Furthermore, if SC were being rapidly metabolized to the OC form, the AUC of OC pDNA after administration of SC pDNA would be nearly equal to the AUC after administration of OC pDNA. However, the AUC of OC was only $60 (\pm 10)$ % of the

AUC obtained after OC, leaving ~40% of the plasmid to be accounted for by entrapment, association, or conversion to a form undetectable using this method.

Open circular pDNA was cleared less rapidly than the SC form of the plasmid. This may be partially due to the accessibility of various nucleases. Open circular plasmid must be nicked by endonucleases on each sister strand in the same location to generate L pDNA. It is reasonable to assume that there are single strand nicks occurring in the OC plasmid during its entire time course of elimination. However, it is not until a second nick is proximal to another nick in the sister strand that degradation of the OC form is detected. Linear pDNA would be degraded by a mechanism similar to the degradation of the OC form, but also by exonucleases acting on the free ends of the plasmid. This additional route of degradation would contribute to L pDNA's more rapid clearance.

Alternatively, the mechanism for the rapid clearance of SC pDNA may be due to physical differences between the 3 forms. Previous studies comparing SC pDNA to L pDNA have shown that SC pDNA has stronger acidity than L pDNA (Poly 1999). This difference is the result of the density and availability of the free phosphate groups. Acidic groups located at the external loops of SC molecules would be available and involved in interactions, while most of the phosphate groups localized within the SC molecule would not interact with components in the bloodstream. OC and L pDNA however likely expose a much higher number of available acidic functional groups (Poly 1999). These anionic charges would be located all along the pDNA molecules and allow for multiple interactions. This decreased binding affinity of SC pDNA has been displayed in interactions with silica (Melzak 1996) and clay minerals (Poly 1999). The SC form of the plasmid has also been shown to interact more strongly with the

hydrophobic stationary phase in reversed-phase high performance liquid chromatography (Colote 1986). This difference in exposed electrostatic groups could potentially explain the rapid clearance of SC pDNA relative to OC and L pDNA. If L and OC pDNA interact more strongly with plasma components than SC pDNA this may decrease their uptake by scavenger receptors or tissues. Furthermore, this association of OC and L pDNA with plasma components may also offer some protection from plasma nucleases. Protection from nucleases has been displayed after adsorption to proteins and is the basis for DNase I footprinting (Lodish 1995). This would result in SC pDNA remaining free in the bloodstream and open to nuclease digestion. Also, the increased hydrophobicity of SC pDNA may also lead to a greater interaction with vascular endothelia, providing an additional clearance pathway, and explaining SC pDNA's larger volume of distribution. Thus, these physical differences may provide some insight into the observed pharmacokinetic differences.

OC pDNA displayed kinetics consistent with saturable elimination. Nonlinear elimination of OC pDNA has previously been suggested using pharmacokinetic analysis of outflow patterns from rat perfused liver studies with radiolabeled OC pDNA (Yoshida 1996). In this study V_d increased and extraction ratio decreased as perfusion dose was increased from 1.33 to 13.3 $\mu\text{g/liver}$. The results presented here contribute further evidence to support this finding.

In conclusion, these results indicate that naked SC pDNA is cleared rapidly from the rat circulation after IV bolus administration at $390 (\pm 50)$ ml/min, and has a volume of distribution of $148 (\pm 26)$ ml. AUC analysis revealed that $60 (\pm 10)$ % of the SC pDNA degraded to the OC form of the plasmid. The OC form of the plasmid exhibits

nonlinear characteristics with clearance ranging from $1.3 (\pm 0.2)$ to $8.3 (\pm 0.8)$ ml/min for the 2500 and 250 μg doses, respectively. V_d of the OC form was $43 (\pm 15)$ ml. The conversion of the OC form of the plasmid to the L form of the plasmid appears to be nearly complete. The L form of the plasmid is cleared at $7.6 (\pm 2.4)$ ml/min and has a V_d of $38 (\pm 12)$ ml.

CHAPTER 5

PHARMACOKINETIC MODELING OF PLASMID DNA AFTER IV BOLUS ADMINISTRATION IN THE RAT

Introduction

The pharmacokinetics of any drug are best studied by simultaneous measurement of the parent drug and all of its pharmacologically active metabolites, especially if they all possess similar pharmacological properties (Garrett 1984). Plasmid DNA exists as three major topoforms. The native (parent) structure of non-damaged pDNA is supercoiled (SC). Single strand nicks in the phosphodiester backbone of the pDNA yield an open circular (OC) form. This metabolite of SC pDNA is still associated with significant activity (~90-100%) (Adami et al. 1998; Niven et al. 1998). Further single strand nicks to the OC pDNA yield linear (L) pDNA, associated with a significant loss of activity (~90%). This process is schematically illustrated in Figure 1-2.

The mathematical description of the plasma concentration-time curve of a drug after administration yields only an equation describing elimination after a given dose. Thus, it is difficult to predict the concentration-time profile of the drug and metabolites after administration of varying doses. A model that quantitatively describes the transformation of the parent drug and its metabolites as a function of dose, may permit correlations with pharmacodynamic activities, and give insight into the mechanisms of action (Garrett 1984).

The terms “linear” and “nonlinear” describe mathematical concepts related to a given plasma concentration-time dataset’s dependence on administered dose (Garrett

1984). Linear differential equations describe definite properties. First, transfers from drug to metabolite are first order. Second, multiples of dose yield the same multiple of drug concentration in any compartment at the same time. Finally, the plasma concentration-time curve can be described by a linear sum of exponentials.

A non-linear model does not exhibit these properties (Garrett 1984). The rate of this type of system is not simply proportional to the plasma concentration. A well-characterized example of this type of system is the Michaelis-Menten model of elimination of a metabolized drug in a one-compartment model. This model is described by the following equation:

$$\frac{dC}{dt} = \frac{V_{\max} \cdot C}{K_m + C}$$

Where C is the parent drug's plasma concentration, K_m is the concentration at which the system operates at half of its maximal velocity, and $V_{\max}$ is the systems maximal velocity. In this model, drug clearance is not constant, but varies with plasma concentration.

In order to properly dose and achieve the desired levels of protein transcript it will be necessary to clearly define the pharmacokinetic parameters involved with the clearance of pDNA from the bloodstream. Therefore it is necessary to construct a mathematical model to predict pDNA concentrations *in vivo*. We have used to the previous information to construct this model. Noncompartmental analysis had suggested that OC pDNA was subject to non-linear elimination. AUC analysis suggested that 60 (± 10) % of the SC pDNA was being converted to the OC form of the plasmid. Furthermore, AUC analysis also suggested complete conversion of the OC form of the plasmid to the L form. SC pDNA was detectable only after a 2500 μg dose. Therefore the parameters for this form of the plasmid were limited to data from this dose only.

These characteristics of the noncompartmental analysis were incorporated into a model describing the elimination of pDNA from the bloodstream.

The aim of this study was to accurately model the pharmacokinetics of pDNA in a topoform specific manner after single dose administration. This information will provide a basis upon which pharmacokinetic/pharmacodynamic models can be constructed. Furthermore, this model may provide useful in analyzing the kinetic effects of pDNA delivery vehicles.

Theoretical

SC and L pDNA concentrations were fit to pseudo first-order kinetics. OC pDNA was fit to Michelis-Menten kinetics. The degradation of L pDNA is considered to yield fragments of heterogeneous lengths, thus these products were not included in the fitted model. The model is presented in Figure 5-1. Based on this model the following differential equations were derived to describe the kinetics of pDNA:

$$\begin{aligned}\frac{dSC}{dt} &= -(k_s + k_u) \cdot SC \\ \frac{dOC}{dt} &= k_s \cdot SC - \frac{V_{\max(OC)} \cdot OC}{(K_{m(OC)} + OC)} \\ \frac{dL}{dt} &= \frac{V_{\max(OC)} \cdot OC}{(K_{m(OC)} + OC)} - k_L \cdot L\end{aligned}$$

Where SC, OC, and L are the amounts of SC, OC, and L pDNA present at time (t) respectively. The constant k_s represents the rate constant for the degradation of SC pDNA to OC pDNA. The constant k_u represents the removal of SC pDNA from the circulation or degradation to an undetectable form. The parameter $V_{\max(OC)}$ is the apparent maximal rate of elimination of OC pDNA from the circulation. The constant $K_{m(OC)}$ represents the apparent concentration at which the kinetics operate at $\frac{1}{2} V_{\max}$. The

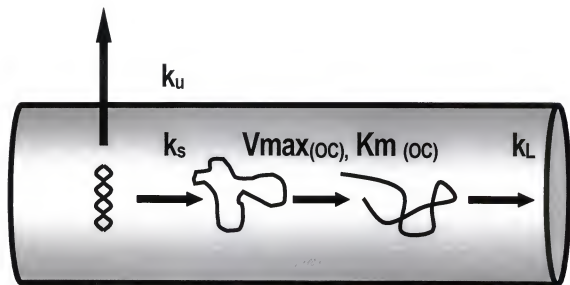


Figure 5-1. Model for pDNA clearance from the bloodstream.

constant k_L represents the first-order rate constant for L pDNA.

The kinetics of OC pDNA after IV bolus of OC pDNA were fit to a Vmax model:

$$\frac{dOC}{dt} = -\frac{V_{\max(OC)} \cdot OC}{K_{m(OC)} + OC}$$

The kinetics of L pDNA after IV bolus of L pDNA were fit to:

$$\frac{dL}{dt} = -k_L \cdot L$$

Non-linear curve fitting and statistical analysis were carried out using Scientist (version 4.0, Micromath, Salt Lake City, UT). Goodness of fit was assessed using model selection criteria (MSC) (MicroMath 1995). Area under the plasma concentration time curve (AUC) was calculated using trapezoidal rule. Pharmacokinetic analysis was carried out using standard pharmacokinetic parameters as noted in the text. Statistical analysis was performed using SAS (Version 6.12, The SAS institute, Cary, NC).

Results

The concentrations of supercoiled pDNA were detectable only after the 2500 µg dose. The parameters for this form of the plasmid were fixed for all other doses. Resulting observed and fitted data for the SC pDNA were presented in Figure 4-6. SC pDNA was eliminated with a $t_{1/2}$ of 0.15 (± 0.01) min.

Plasma concentrations and resulting fitted data for the OC and L pDNA are presented in Figure 5-2 to 5-5 for the 2500, 500, 333, and 250 µg doses, respectively. Calculated pharmacokinetic parameters are presented in Table 5-1. Predicted data agreed well with experimental observations. Model selection criteria were 4.1, 4.3, 3.2, and 4.0 for the 2500, 500, 333, and 250 µg doses, respectively. OC pDNA was eliminated with

an average $V_{\max}$ of $1.7 (\pm 0.5)$ ng/ μ l/min and an average K_m of $7.1 (\pm 2.1)$ ng/ μ l over all doses. L pDNA was eliminated with an average $t_{1/2}$ of $1.7 (\pm 0.5)$ min. Parameters calculated after the data from all doses were fit simultaneously to the model are presented in Table 5-2. This global fitting of the data resulted in an increase in model selection criteria to 4.4.

We next sought to determine if the calculated pharmacokinetic parameters would remain consistent after administration of each form of the plasmid independently versus after administration of the SC form of the plasmid. Thus OC pDNA was administered at 2500 and 250 μ g doses and the concentrations of OC and L pDNA in the bloodstream monitored. Non-linear curve fitting for the OC form of the plasmid was carried out using a model for saturable metabolism as described in the methods.

Predicted concentrations of OC pDNA agreed well with experimental data. Experimental and observed data are presented in Figures 5-6 for the 2500 and 250 μ g doses.

Calculated pharmacokinetic parameters are presented in Table 5-3. The elimination of OC pDNA was more appropriately described by Michaelis-Menten elimination with a statistical improvement of fit observed after utilization of this model. OC pDNA was eliminated with a $V_{\max}$ of $1.0 (\pm 0.3)$ ng/ μ l/min and a K_m of $3.9 (\pm 0.9)$ ng/ μ l. L pDNA was eliminated with an average $t_{1/2}$ of $1.7 (\pm 0.5)$ min after administration of OC pDNA (Figure 5-7).

The L form of the plasmid was also administered at 2500 and 250 μ g doses by IV bolus administration. The concentrations of L pDNA were then monitored. Non-linear

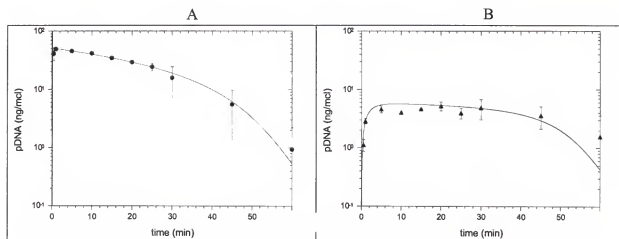


Figure 5-2. Concentrations of (A) OC and (B) L pDNA in the bloodstream after 2500 µg dose of SC pDNA. Data points represent the averages of $n=3 \pm 1$ standard deviation. Lines represent concentrations predicted by the model.

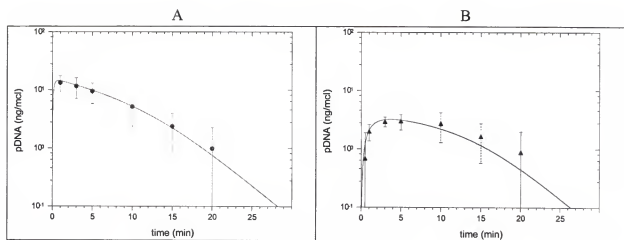


Figure 5-3. Concentrations of (A) OC and (B) L pDNA in the bloodstream after 500 μ g dose of SC pDNA. Data points represent the averages of $n=3 \pm 1$ standard deviation. Lines represent concentrations predicted by the model.

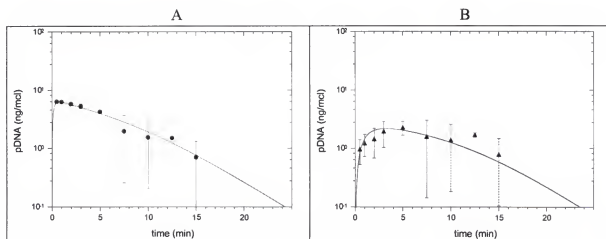


Figure 5-4. Concentrations of (A) OC and (B) L pDNA in the bloodstream after 333 µg dose of SC pDNA. Data points represent the averages of $n=3 \pm 1$ standard deviation. Lines represent concentrations predicted by the model.

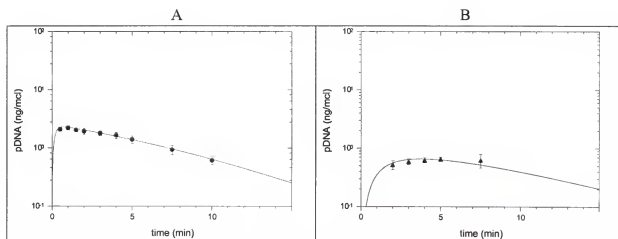


Figure 5-5. Concentrations of (A) OC and (B) L pDNA in the bloodstream after 250 μg dose of SC pDNA. Data points represent the averages of $n=3 \pm 1$ standard deviation. Lines represent concentrations predicted by the model.

Table 5-1. Pharmacokinetic parameters for pDNA based upon the model presented in the text.

Parameter	2500 μg	500 μg	333 μg	250 μg
$k_u(\text{min}^{-1})$	1.9 (± 0.1)	-	-	-
$k_s(\text{min}^{-1})$	2.8 (± 0.2)	-	-	-
$V_{\max}(\text{ng}/\mu\text{l}/\text{min})$	1.3 (± 0.1)	2.0 (± 0.4)	2.1 (± 0.6)	1.3 (± 0.4)
$K_m(\text{ng}/\mu\text{l})$	6.1 (± 0.8)	8.8 (± 3.7)	7.9 (± 0.7)	5.7 (± 0.6)
$k_t(\text{min}^{-1})$	0.39 (± 0.02)	0.42 (± 0.01)	0.42 (± 0.03)	-
MSC	4.3 (± 1.4)	3.4 (± 1.4)	3.6 (± 0.5)	3.0 (± 0.6)

Parameters represent averages of $n=3 \pm 1$ standard deviation.

Table 5-2. Overall pharmacokinetic parameters for pDNA when all doses are fit simultaneously.

Parameter	Value
$V_{\max}$ (ng/ μ l/min)	1.4 ($\pm$ 0.1)
K_m (ng/ μ l)	7.2 ($\pm$ 1.9)
k_t (min ⁻¹)	0.43 ($\pm$ 0.16)
MSC	4.4

Parameters represent simultaneously fitted values ($\pm$ standard deviation of the fit) from means of pDNA concentrations in the bloodstream after administration of SC pDNA at 2500, 500, 333, and 250 μ g doses.

curve fitting for the L form of the plasmid was carried out using a one compartment model with first-order elimination as described in the methods section.

Predicted concentrations of L pDNA agreed well with experimental data. Experimental and observed data for L pDNA are presented in Figures 5-8 for the 2500 and 250 μg doses. Calculated pharmacokinetic parameters for L pDNA are presented in Table 5-4. L pDNA was eliminated with a $t_{1/2}$ of $2.15 (\pm 0.15)$ min.

Conclusions

Noncompartmental analysis had suggested several different characteristics of pDNA pharmacokinetics. First it had suggested that the conversion of SC to OC pDNA was not a complete process. Instead 60 % of the SC pDNA appeared to be converted to the OC form of the plasmid. The results of the curve fitting experiments further supported this relationship with k_s able to be fit to 60 % of the overall elimination of SC plasmid (i.e. the sum of k_s and k_u).

A weakness of this model is that the SC form of the plasmid was detectable only after the 2500 μg dose. Thus, we were forced to assume linear pharmacokinetics for this form of the plasmid. It is true that this elimination could occur more rapidly at lower doses. However, the rate at the 2500 μg dose is already so rapid, half-life = $0.15 (\pm 0.02)$ min, that it is unlikely that fixing this parameter would significantly affect the calculated values for the OC and L form of the plasmid.

Secondly, the noncompartmental analysis suggested non-linear processes were involved in the elimination of the OC plasmid from the bloodstream. Curve fitting to first order parameters also resulted in similar suggestions between doses. The value of the first order rate constant for OC elimination ranged from 0.035/min to 0.14/min

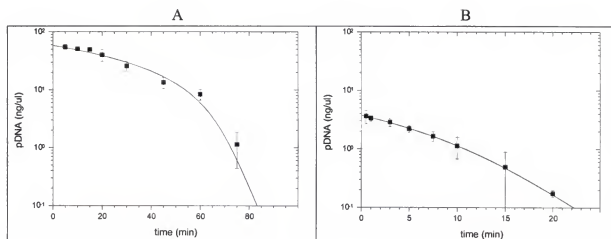


Figure 5-6. Concentrations of OC pDNA in the bloodstream after (A) 2500 μ g and (B) 250 μ g dose of OC pDNA. Data points represent the averages of $n=3 \pm 1$ standard deviation. Lines represent concentrations predicted by the model.

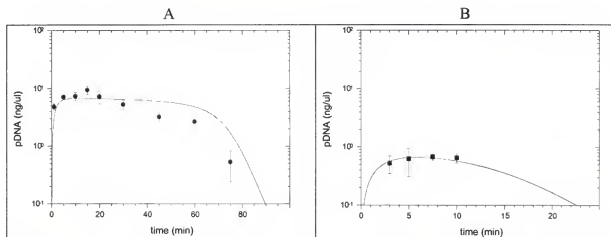


Figure 5-7. Concentrations of L pDNA in the bloodstream after (A) 2500 µg and (B) 250 µg dose of OC pDNA. Data points represent the averages of $n=3 \pm 1$ standard deviation. Lines represent concentrations predicted by the model.

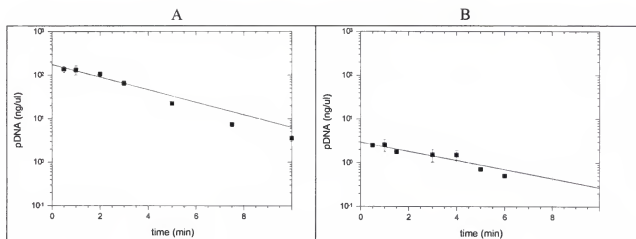


Figure 5-8. Concentrations of L pDNA in the bloodstream after (A) 2500 µg and (B) 250 µg dose of L pDNA. Data points represent the averages of $n=3 \pm 1$ standard deviation. Lines represent concentrations predicted by the model.

Table 5-3. Pharmacokinetic parameters calculated after administration of OC pDNA at 2500 and 250 μg doses.

Parameter	2500 μg Dose OC	250 μg Dose OC
$V_{\max}$ ($\text{ng}/\mu\text{l} \cdot \text{min}$)	1.1 (± 0.1)	0.90 (± 34)
K_m ($\text{ng}/\mu\text{l}$)	4.4 (± 0.4)	3.4 (± 1.1)
k_1 (min^{-1})	0.39 (± 0.12)	0.49 (± 0.07)
MSC	4.4 (± 0.4)	3.4 (± 1.1)

Parameters represent mean of $n=3 \pm 1$ standard deviation.

Table 5-4. Pharmacokinetic parameters calculated after administration of L pDNA at 2500 and 250 μg doses.

Parameter	2500 μg Dose L	250 μg Dose L
k_1 (min^{-1})	0.32 (± 0.02)	0.33 (± 0.03)
MSC	2.4 (± 0.6)	2.1 (± 0.9)

Parameters represent mean of $n=3 \pm 1$ standard deviation.

between the 2500 and 250 μg dose data sets, respectively. Thus, it was necessary to include non-linear elimination into the model to describe this change in elimination between doses.

Non-linear elimination of pDNA has previously been suggested using pharmacokinetic analysis of outflow patterns from rat perfused liver studies with radiolabeled OC pDNA (Yoshida 1996). In this study volume of distribution decreased, as perfusion dose was increased from 1.33 to 13.3 $\mu\text{g}/\text{liver}$, from 0.598 (± 0.09) to 0.314 (± 0.08) ml/g respectively. Extraction percentage decreased from 45.56 (± 0.31) to 20.12 (± 0.75) % as dose ranged from 1.33 to 13.3 $\mu\text{g}/\text{liver}$ respectively. Thus, the results presented here contribute further evidence to support nonlinear processes in the elimination of OC pDNA from the circulation.

The values of V_{max} and K_m remained relatively constant between doses of SC pDNA and also after administration of OC versus after administration of SC pDNA. After administration of SC pDNA, the average of all doses for V_{max} was 1.7 (± 0.5) versus 1.0 (± 0.3) $\text{ng}/\mu\text{l}/\text{min}$ after administration of OC pDNA. Likewise, the value of k_L remained relatively constant after administration of all 3 forms of the plasmid. The value of k_L ranged from 0.47 (± 0.11) to 0.45 (± 0.10) to 0.32 (± 0.02) min^{-1} after administration of SC, OC, or L pDNA respectively.

The presented data were all weighted with a factor of 0. This has resulted in some of the lower concentration data points being less well approximated by the model than higher concentrations. More sensitive quantitation techniques may allow the use of weighting factors in the fitting and provide better predictions of lower concentration data.

Thus, we conclude from the modeling experiments that SC pDNA is rapidly converted to the OC form of the plasmid with a half-life of $0.15 (\pm 0.01)$ min. OC pDNA exhibits non-linear characteristics with a $V_{\max}$ of $1.5 (\pm 0.6)$ ng/ μ l/min and K_m of $6.0 (\pm 2.4)$ ng/ μ l. The L form of the plasmid exhibits first-order kinetics and is eliminated with an overall average half-life of $1.6 (\pm 0.4)$ min.

CHAPTER 6 PHARMACOKINETICS OF LIPOSOME: PLASMID DNA COMPLEXES

Introduction

One of the major obstacles to effective gene therapy is the generally poor efficiency of pDNA delivery (Thierry et al. 1997). Most gene therapy efforts involve the use of retroviral vectors due to their efficiency and stable integration (Thierry et al. 1997). Clinical use of retroviral vectors, is however faced with a large number of obstacles. Among these are laborious preparation, difficulties in purification, concerns for the recombination with endogenous virus to produce a potentially infectious virion, and integration into the host genome to produce a tumorigenic or cytotoxic event (Liu 1999). Because of these limitations, the use of non-viral techniques, including liposomal delivery, has become an intensely investigated area. An early published report displaying incorporation of pDNA into liposome: pDNA complexes speculated that "...possibly such liposomes could be used as vehicles for the introduction of new genes into cells" (Osaka et al. 1996). This report was followed by a large number of studies demonstrating successful *in vitro* liposome-mediated transgene expression in prokaryotic and eukaryotic cells (Osaka et al. 1996). Cationic lipids have been shown to be safe and are currently being utilized in gene therapy clinical trials (Valere 1999).

The data from the previous studies presented here, display that the circulation time of pDNA in the circulation is remarkably short, with a rapid conversion from the SC form of the plasmid to the OC and L forms. This change in topoform has been shown to affect transcriptional activity (Murray 1991; Niven et al. 1998). For successful gene

therapy, longer circulation times of the more transcriptionally active SC and OC forms of the plasmid would likely be beneficial. Furthermore, liposomal complexation with plasmid DNA offers the advantage of conjugation of a targeting ligand. Thus this may allow pDNA to be directed to the desired site of action.

Although few studies are available on the pharmacokinetics of liposome:pDNA complexes, liposomal pharmacokinetics alone have been studied extensively with several reviews published (Hwang et al. 1997; Juliano 1988; Takakura et al. 1996). Liposome pharmacokinetics have been shown to be dependent upon size (Sato 1986), dose (Bosworth and Hunt 1982; Osaka et al. 1996), lipid composition (Gabizon 1988), and charge (Juliano 1988). In general, liposomes larger than 60 nm in diameter are unable to access tissues having continuous capillary endothelia, including skeletal, cardiac, and smooth muscle, lung, skin, subcutaneous tissue, and serous and mucous membranes, and are limited to uptake in tissues of the reticuloendothelial system (Hwang et al. 1997). Liposomes larger than 0.5 μm are confined to the vasculature in all tissues.

After systemic administration of liposome:pDNA complexes, pDNA rapidly disappears from the bloodstream. Niven and coworkers (Niven et al. 1998) showed that 2.9 % of the dose of liposome:pDNA complexes could be recovered in the bloodstream 5 minutes after administration. Plasmid remained detectable ($\leq 2\%$ of the dose recovered) in the bloodstream through 24 h after administration. The clearance processes involve degradation in the blood stream (as displayed in the previous studies), interaction with plasma proteins, organ distribution, and uptake by the reticuloendothelial system (Juliano 1988). The movement of liposome:pDNA complexes into tissues has been suggested to

be roughly a unidirectional system, where distribution back into the central compartment can be assumed to be negligible (Mahato et al. 1997).

Previous studies had suggested that liposome: pDNA complexes offered protection of the pDNA from degradation by plasma nucleases (Thierry et al. 1997), but pDNA was removed from the circulation in a more rapid fashion than after administration of the naked plasmid (Osaka et al. 1996; Niven et al. 1998). Niven and coworkers (Niven et al. 1998), using [^{33}P]pDNA, found that 36 % of the pDNA dose could be recovered in the bloodstream at 5 min after administration of naked pDNA and only 2.9 % of the dose could be recovered at 5 min after administration of liposome: pDNA complexes. Osaka and coworkers (Osaka et al. 1996) also found similar results 2 minutes after administration of liposome [^{33}P]pDNA complexes with 6.12 % of dose equivalents/g in the blood after administration of liposome:pDNA complexes versus 15.79 % of dose equivalents/g after administration of free [^{33}P] pDNA.

A large number of cationic lipids have been synthesized since the initial report in 1987 (Liu et al. 1997). *In vitro* the incorporation of the neutral phospholipid dioleoylphosphatidylethanolamine (DOPE) into 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) liposomes helps to destabilize the endocytic vacuole membrane allowing the release of exogenous DNA into the cytosol (Osaka et al. 1996), and represents a commonly used lipid mixture. While *in vivo*, the use of DOTAP: cholesterol liposomes is more common (Barron et al. 1998). Thus, these 2 lipid combinations were chosen to study the effects of liposome complexation on the pharmacokinetics of pDNA.

The objective of these studies was to investigate the potential protective effects of liposome complexation on preservation of the SC topoform. We began by initially

studying, *in vitro*, the protective effects of liposome complexation in isolated plasma. We next analyzed the effect of liposome:pDNA ratio on this protection. Finally we sought to compare the effects of liposome complexation on the *in vivo* pharmacokinetics of pDNA with the pharmacokinetics of naked pDNA at equivalent dose.

Methods

All chemicals used were obtained similar to their description in Chapter 2 Methods. Plasmid DNA was obtained as described in Chapter 2 Methods. All lipids were purchased from Avanti Polar Lipids (Alabaster, AL).

For *in vivo* experiments, liposomes were prepared by mixing 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and cholesterol in a 1:1 molar ratio in chloroform and drying the mixture under nitrogen at 60°C in a Buchi Rotovapor. The dried lipid film was then reconstituted in sterile water containing 5% dextrose at a total lipid concentration of 5 mg/ml and shaken in a 60°C water bath for 15 min. Liposomes were then sized by sonication with an ultrasonic probe (Fisher Scientific, Springfield, NJ). Liposome: pDNA complexes were formed by mixing liposomes with pDNA at a 2:1 lipid: DNA weight ratio and incubating at room temperature for 15 min.

For *in vitro* experiments, lipid: pDNA complexes were formed using 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and dioleoyl phosphatidylethanolamine (DOPE) in a 1:1 molar ratio. Lipid: pDNA complexes were formed by complexing pGE150 with DOTAP: DOPE liposomes for 15 min prior to beginning the experiment. Complexes were formed at 1:1, 3:1 and 6:1 lipid: DNA ratios (w/w). Complexes were then incubated in rat plasma and samples drawn at various time points as described previously. Statistical analysis was carried out using SAS (Version 6.12, SAS, Cary, NC) and a two tailed equal variance student's t-test.

For *in vitro* experiments, blood was isolated from male Sprague-Dawley rats (300-350g) by cardiac puncture, and immediately placed in heparinized test tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) on ice. Blood samples were centrifuged at 6,000 g for 5 min. 600 μ l of plasma was removed and placed on ice until assay. Plasma samples were warmed to 37° C in a water bath, and maintained at 37° C for the duration of the experiment. 12 μ g of pDNA, in phosphate buffered saline, was incubated in the 37° C plasma and 50 μ l samples were taken at the times indicated. 80 μ l of phenol: chloroform: isoamyl alcohol (25: 24: 1, v/v/v) was immediately added to each sample, vortexed for 5 s at low speed, and placed on ice. Samples were centrifuged at 20,800 g for 10 min at room temperature. From the supernatant, an aliquot of 15 μ l was removed, 5 μ l of 1 x loading dye (Promega, Madison, WI) added, and placed on ice until loaded on an agarose gel. A final volume of 10 μ l was loaded on agarose gels. Analysis and quantitation was performed as described in Chapter 2 Methods.

IV administration and analysis of liposome: pDNA complexes were performed as described earlier in Chapter 3 methods. Briefly, SC liposome: SC pDNA complexes were administered to male Sprague-Dawley rats (300-350 g) by IV bolus in the femoral vein at a dose equivalent to 500 μ g of pDNA. Blood samples were drawn through a jugular vein cannula. Isolated blood samples (approximately 300 μ l) were immediately placed in tubes containing 57 μ l of 0.34mM EDTA. Samples were then liquid: liquid extracted with phenol: chloroform: isoamyl alcohol (25: 24:1 v/v/v). Quantitative analysis was performed as described earlier in Chapter 3 methods.

Results

Pharmacokinetics of Liposome:pDNA Complex Degradation in Rat Plasma

To investigate the potential protective effects of cationic liposomes from plasma nucleases, we complexed a common liposomal delivery vehicle, DOTAP: DOPE (1:1 w/w), to pDNA (3:1 lipid: DNA, w/w) and incubated the complexes in freshly isolated rat plasma at 37°C. Figure 6-1 displays that a portion of the SC pDNA remained detectable through 5.5 h (8.2% of the time=0 amount).

We next sought to determine the effect of lipid: pDNA ratio on the degradation observed in the previous experiment. We hypothesized that increasing the lipid:pDNA ratio should offer increased protection from nucleases. We chose to investigate this by complexing lipid: pDNA at 1: 1, 3:1, and 6:1 ratios. Agarose gel analysis revealed that the SC pDNA was detectable through at least 5 hours in all three ratios (Figure 6-2). Quantification of the percent SC remaining revealed that 28.9%, 37.8%, and 17.7% (for 6:1, 3:1, and 1:1 respectively) of the 1 minute amount remained at 3 hours. A statistical analysis of the percent SC plasmid remaining at revealed that there was no statistically significant difference between the percent remaining in the 6:1 versus 1:1, 3:1 versus 1:1 and the 6:1 versus 3:1 ratios. These results suggest increasing the lipid: DNA ratio from 1:1 to 6:1 offers no significant increase in protection from plasma nucleases through 3 hours.

The OC and L forms of the plasmid were hypothesized to appear for three possible reasons. First, it is possible that some portion of the pDNA remains non-complexed and free in solution. When this mixture of complexed pDNA and free pDNA is incubated in the plasma, the free pDNA degrades as previously described, and thus the OC and L pDNA appear and degrade. A second possible explanation is that the pDNA

on the outer surface of the liposome: pDNA complex aggregates is offered little protection from plasma nucleases and is degraded. The pDNA towards the core of the complex is then spared from degradation. Finally, it is possible that the complexes degrade in the plasma.

To confirm that all pDNA was complexed, we complexed lipid: pDNA at 1:1, 3:1, and 6:1 ratios (w/w) and analyzed their migration through an agarose gel. If any pDNA remained non-complexed, it should migrate through the gel and separate from the complexed pDNA remaining in the well. No migration from the wells was observed at any lipid: pDNA ratio as seen in Figure 6-3. These results suggest that all pDNA is complexed at all three ratios, pDNA must therefore be degrading from the surface of the liposomes or the complexes must dissociate in the plasma.

Pharmacokinetics of Liposome:pDNA Complex Degradation in Rat Whole Blood

We next sought to determine if the effects we observed using liposomes in the plasma were consistent with the effects we would observe in whole blood. Unlike naked pDNA, liposome/pDNA complexes have been shown to be recovered in the red blood cells (37 to 84 %) (Osaka et al. 1996). We hypothesized that this uptake may affect the kinetics observed. Liposome/pDNA complexes taken up by the red blood cells may be disassembled in the RBC and pDNA degraded when freed from the protective liposome complex. Thus our hypothesis was that pDNA would break down faster in whole blood. We also sought to determine the effects of increasing the lipid:pDNA ratio on this process, and secondarily hypothesized that increasing the lipid:pDNA ratio should lead to more rapid uptake by RBC, resulting in more rapid degradation of the pDNA.

The results of the whole blood experiments revealed that the protocol used in isolation of pDNA from the plasma also were sufficient to isolate pDNA from whole

blood. Plasmid DNA incubated in whole blood did not degrade faster than pDNA in the plasma (Figure 6-4 A). Furthermore, increasing the lipid:pDNA ratio from 3:1 to 6:1 did not serve to increase the degradation observed (Figure 6-4 B).

These results suggest that the presence of RBC does not significantly affect the degradation rate observed. An increase in lipid:pDNA ratio also does not affect the degradation observed in the plasma. Liposome complexation results in a relative maintenance of the SC topoform with 28.9%, 37.8%, and 17.7% (for 6:1, 3:1, and 1:1 respectively) of the 1 minute amount remaining after 3 hours.

Pharmacokinetics of Liposome:pDNA Complexes after IV Bolus Administration in the Rat

The *in vitro* results displayed that liposome complexation can protect a portion of the SC topoform from degradation through 5.5 hours. We next sought to determine if liposome complexation would provide similar protection *in vivo*. Thus, we injected liposome: SC pDNA complexes at a 500 μ g dose of pDNA as described in the methods section.

Agarose gel analysis obtained after administration of liposome: pDNA complexes is presented in Figure 6-5. Plasma concentrations of SC, OC, and L pDNA are presented in Figure 6-6. Unlike after administration of naked pDNA, SC pDNA was readily detectable after administration, and remained detectable through 5 minutes after administration of the 500 μ g dose. However, the OC and L form of the plasmid also only remained detectable through 5 min after administration, versus through 20 minutes after administration of naked pDNA.

Noncompartmental analysis of all three forms of the plasmid is presented in Table 6-1, and a relative comparison of the naked and liposome complex parameters is

presented in Tables 6-2, 6-3, and 6-4 for SC, OC, and L pDNA, respectively. If we again make the assumption that SC pDNA follows linear pharmacokinetics, mean residence time for the SC form of the plasmid increased from 0.21 ($\pm$ 0.02) after administration of naked pDNA to 0.99 ($\pm$ 0.42) min after administration of liposome:SC pDNA complexes. Clearance decreased from 390 ($\pm$ 10) to 87 ($\pm$ 30) ml/min. Obviously this disparity would only become more severe if the true pharmacokinetics of naked SC pDNA are more rapid than this at the 500 μ g dose. Area under the concentration time curve of the OC form of the plasmid decreased after liposome complexation from 120 ($\pm$ 50) to 14 ($\pm$ 4) ng/ μ l*min. Clearance/f of the OC form of the plasmid increased from 6.9 ($\pm$ 2.8) to 95 ($\pm$ 37) ml/min. Area under the concentration time curve (AUC) of L pDNA also decreased from 52 ($\pm$ 25) to 5.7 ($\pm$ 1.9) ng/ μ l*min.

Conclusions

These results indicate that liposome complexation can indeed offer protection of the SC form of the plasmid. This increase in circulation half-life provides further evidence to support the use of liposomal delivery vehicles in gene therapy. This analysis also provides evidence that, although the liposome complexation does protect the SC form of the plasmid, the individual particles have an overall more rapid clearance from the circulation. The OC and L form of the plasmid also only remained detectable through 5 min after administration, versus through 20 minutes after administration of naked pDNA.

This finding was mirrored by the [^{33} P]DNA results of Niven and coworkers (Niven et al. 1998) who found that 36 % of the pDNA dose could be recovered in the bloodstream at 5 min after administration of naked pDNA and only 2.9 % of the dose

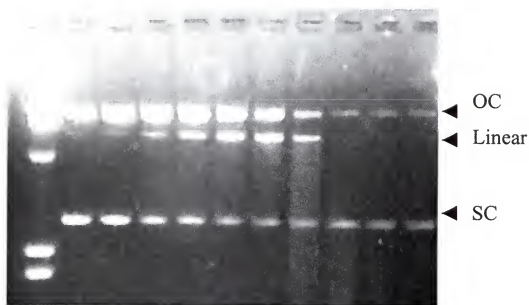


Figure 6-1. Liposome-pDNA complexes were incubated in rat plasma for various time points. 10 μ l of sample was loaded in each lane as described in the methods section. Lane 1; size standard, lane 2; 1 min, lane 3; 2 min, lane 4; 5 min, lane 5; 10 min, lane 6; 20 min, lane 7; 30 min, lane 8; 60 min, lane 9; 2 h, lane 10; 3 h, lane 11; 5.5 h.

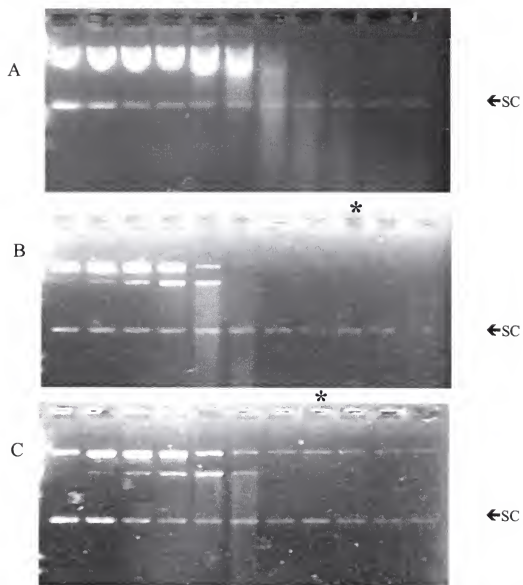


Figure 6-2. Agarose gel analysis of liposome/pDNA complexes. (A) 1:1 lipid:pDNA ratio, through 4 hours. (B) 3:1 lipid:pDNA ratio, through 6 hours. (C) 6:1 lipid:pDNA ratio, through 6 hours. *Indicates the 3 hour time point.

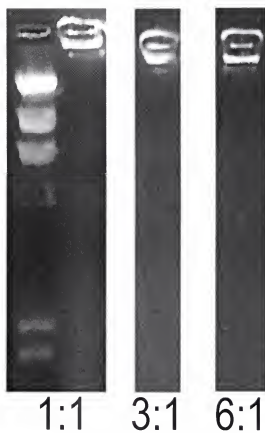


Figure 6-3. Lane 1: high molecular weight size standard, lane 2: 1:1 lipid:pDNA complexes, lane 3: 3:1 lipid:pDNA ratio (w/w), lane 4: 6:1 lipid:pDNA ratio

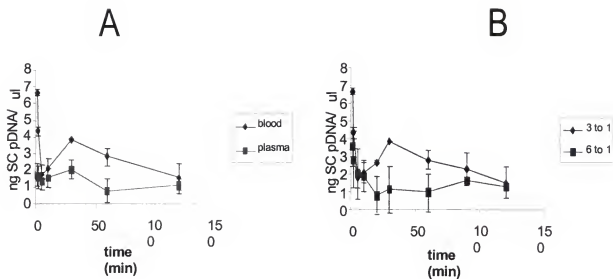


Figure 6-4. (A) Degradation of SC pDNA in rat blood versus plasma. (B) Degradation of supercoiled pDNA in 3:1 and 6:1 (w/w) liposome/pDNA complexes incubated in heparinized rat whole blood. Error bars indicate standard deviation of $n=3$ rats.



Figure 6-5. Agarose gel analysis of pDNA after administration of liposome: pDNA complexes. Lane 1: 15 sec, lane 2: 30 sec, lane 3: 45 sec, lane 4: 1 min, lane 5: 1.5 min, lane 6: 2 min, lane 7: 2.5 min, lane 8: 3 min, lane 9: 4 min, lane 10: 5 min.

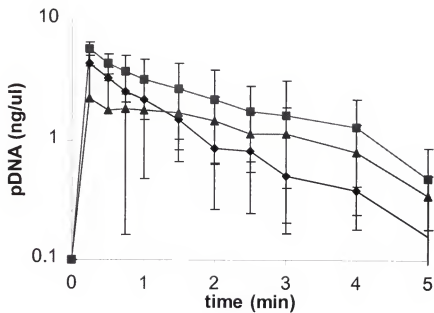


Figure 6-6. Plasma concentrations of SC, OC, and L pDNA after 500 μ g IV bolus administration of SC pDNA: liposome complexes. Key: $\blacklozenge$: SC, $\blacksquare$: OC, $\blacktriangle$: L.

Table 6-1. Noncompartmental analysis of pDNA after administration of liposome: SC pDNA complexes.

Parameter	Supercoiled	Open Circular	Linear
AUC (ng/ μ l*min)	0.0063 ($\pm$ 0.0023)	0.014 ($\pm$ 0.004)	0.0057 ($\pm$ 0.0019)
AUC % extrapolated	12 ($\pm$ 3)	25 ($\pm$ 18)	68 ($\pm$ 26)
AUMC (ng/ μ l*min ²)	0.0068 ($\pm$ 0.0053)	0.04 ($\pm$ 0.004)	0.017 ($\pm$ 0.003)
MRT (min)	0.99 ($\pm$ 0.42)	3.0 ($\pm$ 0.9)	3.3 ($\pm$ 1.0)
Cl/f (ml/min)	87 ($\pm$ 30)	37 ($\pm$ 9)	95 ($\pm$ 37)

Parameters represent averages of $n=3 \pm 1$ standard deviation.

Table 6-2. Comparison of SC pDNA pharmacokinetic parameters after administration of SC pDNA either in free form (naked) at 2500 µg dose or after administration as liposome: pDNA complexes at 500 µg dose.

Parameter	Naked SC pDNA	Liposome: SC pDNA complexes
MRT (min)	0.21 (+0.02)	0.99 (± 0.42)
Cl (ml/min)	390 (±10)	87 (± 30)
Vdss (ml)	148 (±26)	79 (± 16)

Parameters represent averages of $n=3 \pm 1$ standard deviation.

Table 6-3. Comparison of OC pDNA pharmacokinetic parameters after administration of SC pDNA either in free form (naked) or after administration as liposome: pDNA complexes at 500 µg pDNA dose.

Parameter	OC pDNA after Naked SC pDNA	OC pDNA after Liposome:SC pDNA Complexes
AUC (ng/µl*min)	120 (±50)	14 (± 4)
AUC % extrapolated	9 (±4)	25 (± 18)
AUMC (ng/µl*min ²)	1900 (±1200)	40 (± 4)
MRT (min)	15 (±4)	3.0 (± 0.9)
Cl/f (ml/min)	3.0 (±1.2)	37 (± 9)
C _{max} (ng/µl)	13 (±4)	5.8 (± 0.7)
T _{max} (min)	1 (±0)	0.25 (±0)

Parameters represent averages of n=3 ± 1 standard deviation.

Table 6-4. Comparison of L pDNA pharmacokinetic parameters after administration of SC pDNA either in free form (naked) or after administration as liposome: pDNA complexes at 500 µg pDNA dose.

Parameter	L pDNA after naked SC pDNA	L pDNA after Liposome: SC pDNA complexes
AUC (ng/µl*min)	52 (±25)	5.7 (± 1.9)
AUC % extrapolated	15 (±5)	68 (± 26)
AUMC (ng/µl*min ²)	570 (±370)	17 (± 3)
MRT (min)	10 (±2)	3.3 (± 1.0)
Cl/f (ml/min)	6.9 (±2.8)	95 (± 37)
Cmax (ng/µl)	3.2 (±1.0)	1.8 (± 0.8)
Tmax (min)	5.3 (±4.0)	0.25 (±0)

Parameters represent averages of n=3 (± 1 standard deviation).

could be recovered at 5 min after administration of liposome: DNA complexes. Osaka and coworkers (Osaka et al. 1996) also found similar results 2 minutes after administration of liposome [^{33}P]DNA complexes with 6.12 % of dose equivalents/g in the blood versus 15.79 % of dose equivalents/g after administration of free [^{33}P] DNA.

Thierry and coworkers (Thierry et al. 1997) utilized lipospermine (DOGS) and DOPE liposomes and also were able to display protection of pDNA from degradation. In this study SC pDNA was detected for up to 60 min after incubation in isolated plasma, but was rapidly eliminated when IV delivered. Thierry estimated the plasma half-life of the OC pDNA to be much longer than the results presented here at 10 to 20 minutes. Their work did however, show for the first time, the presence of intact pDNA in plasma and blood cells following systemic administration. Lew and coworkers (Lew et al. 1995) used 1,2-dimyristoyl-oxypropyl-3-dimethyl ammonium bromide: DOPE liposomes and determined a half-life of a few minutes for the OC form of the pDNA, similar to the results presented here. However, unlike the results presented here, they reported no detectable SC pDNA. It may be that cationic liposome: pDNA complexes may bind to serum components, and these interactions may differ with different lipids. Indeed, altering lipids and lipid to lipid weight ratios does affect transfection levels of various organs *in vivo* (Liu 1997; Song et al. 1997).

Liu and coworkers (Liu et al. 1997) showed that higher cationic lipid to pDNA ratio was essential to achieve better gene delivery efficiency *in vivo*. The results presented in our studies, however, displayed that increasing the lipid to pDNA ratio from 1:1 to 6:1 had no effect on the observed protection from plasma nucleases in isolated

plasma or whole blood. However, analysis of Liu and coworkers results display that transfection activity remained relatively stable between the 1:1 and 6:1 ratios. Ratios higher than 24:1 were essential to achieve significant increases in transfection activity. Thus, even higher ratios of lipid: pDNA may provide protection from plasma nucleases.

Alternatively, one must also consider the dilution effect in the bloodstream after administration of such a necessarily large volume at these higher lipid: pDNA ratios. It is likely that mouse vasculature is occluded with these high volumes. Thus, complexes travel in the vasculature with little mixing with blood. This would obviously provide protection of the pDNA. However, given that these volumes on a weight basis would not be used in clinical trials in humans (Valere 1999), it is unlikely that this vascular occlusion effect would be so pronounced.

Furthermore, there is likely a maximal amount of complex that can be taken up by tissues. If there is a maximal amount that can be taken up, then there would be a saturation lipid dose that, above which, no further increases in transfection would be observed. This, in fact, was observed in Song and coworkers (Song et al. 1997) and Liu and coworkers (Liu et al. 1997) experiments. Song and coworkers found that ratios from 36:1 to 48:1 offered no increase in transfection, while increases from 2:1 to 36:1 offered significant increases. Liu and coworkers found that ratios between 24:1 and 48:1 also offered no increase in transfection, while increasing the ratio from 6:1 to 24:1 offered significant increases. Thus, these extremely high ratios of lipid: pDNA may be unnecessary to provide adequate protection of the pDNA.

The OC and L pDNA were cleared from the circulation more rapidly than after administration as naked pDNA. This may be due to the fact that liposome pDNA

complexes are more rapidly cleared from the circulation than naked pDNA, regardless of the pDNA form. If this were true, one would predict that administration of radiolabeled pDNA as liposome: pDNA complexes should show higher and more rapid increases of tissue radioactivity than after administration as naked pDNA. This results has been observed after administration of [^{33}P] pDNA (Osaka et al. 1996; Liu 1997; Song et al. 1997; Niven et al. 1998). This was evident as increases in AUC maximal radioactivity recovery/ tissue weight. Blood radioactivity exhibited larger AUCs' and C_{max} s' after administration of naked pDNA. This provides further evidence to support this hypothesis.

In conclusion, liposome pDNA complexes are eliminated from the circulation more rapidly than naked pDNA, while providing protection of the SC topoform from degradation in the bloodstream. This level of protection is independent of lipid: pDNA ratio from 1:1 through 6:1. SC pDNA is detectable through 5 min after administration as liposome: pDNA complexes at a 500 μg dose, whereas it is undetectable after administration as naked pDNA at this dose. Clearance of the SC pDNA decreased from 390 (± 10) to 87 (± 30) after administration as naked or liposome complexes, respectively. Volume of distribution decreased from 148 (± 26) to 79 (± 16) ml after administration as naked or liposome complexes, respectively. OC and L pDNA exhibited decreases in C_{max} after administration as liposome: SC pDNA complexes from 13 (± 4) to 5.8 (± 0.7) ng/ μl and 3.2 (± 1.0) to 1.8 (± 0.8) ng/ μl , respectively. Decreases in t_{max} after administration as liposome: SC pDNA complexes were also displayed for both OC and L pDNA from 1 (± 0) to 0.25(± 0) and 5.3 (± 4.0) to 0.25 (± 0) min, respectively. Clearance/f increased after administration as liposome: SC pDNA complexes for both the OC and L

forms of the plasmid from $3.0 (\pm 1.2)$ to $37 (\pm 9)$ and $6.9 (\pm 2.8)$ to $95 (\pm 37)$ ml/min, respectively. Thus, liposome pDNA complexes are eliminated from the circulation more rapidly than the naked pDNA, while providing protection of the SC topoform from degradation in the bloodstream.

CHAPTER 7 CONCLUSIONS AND IMPLICATIONS

Summary of Results

Implications of Plasmid DNA Degradation in Isolated Plasma

DNase I is a well-characterized enzyme in human plasma present at concentrations averaging 26.1 (± 9.2) ng/ml in the sera of healthy humans (Chitrabamrung 1981). Traditionally the presence of this enzyme has led to the conclusion that pDNA administered IV is degraded in a rapid fashion (Gosse et al. 1965; Chused 1972). This has led to the current view of gene delivery, in which protection from plasma nucleases is a major goal of delivery vehicles. The results of this study reveal that although the half-life of SC and OC pDNA is remarkably short, degradation alone was not enough to explain the rapid disappearance of pDNA from the circulation observed *in vivo*. After IV bolus the rate of degradation of SC pDNA was greater than 7 times faster than in isolated plasma.

Previous reports on the pharmacokinetics of pDNA have only been qualitative, or involved radiolabeling. These studies indicated that pDNA degrades within 5 minutes after incubation in whole blood *in vitro* or after IV injection in mice (Kawabata et al. 1995; Thierry et al. 1997). We sought to quantitatively model the pharmacokinetics underlying the stability of pDNA in the plasma using isolated rat plasma as a model system.

The results presented in Chapter 2 revealed that SC pDNA degrades in isolated plasma with a half-life of 1.2 min. Open circular pDNA is more stable than the

supercoiled topoform degrading with a half-life of 21 min. Linear pDNA is degraded more rapidly than the OC topoform with a half-life of 11 min. A schematic representation of the kinetics of pDNA degradation in isolated rat plasma is presented in Figure 7-1.

Liposome complexation revealed a relative maintenance of the SC topoform through 5.5 h. This provides further evidence to suggest that liposome complexation may not only be a means by which to deliver pDNA to target sights, but also to specifically protect SC pDNA from degradation.

In summary the *in vitro* work presents a pharmacokinetic model describing the degradation of pDNA in rat plasma. Using the model derived, we are able to conclude that naked supercoiled pDNA degrades in rat plasma with a half-life of $1.2 (\pm 0.1)$ min, open circular with a half-life of $21 (\pm 1)$ min, and linear pDNA with a half-life of $11 (\pm 2)$ min. Furthermore, these studies provide evidence that supercoiled pDNA can remain stable in the plasma through 5.5 hours when complexed to cationic liposomes. This degradation was independent of sequence between the pGL3, pGE150 and pGeneMax-Luciferase plasmids.

Comparison of *In Vitro* and *In Vivo* Pharmacokinetics

The results presented in Chapter 3 indicate that SC pDNA was undetectable after IV bolus administration of a 500 μ g dose, whereas SC pDNA was readily detectable in isolated plasma, and remained detectable through 3 min of incubation. Similar results were seen for the OC and L forms of the plasmid. The terminal half-lives of OC and L pDNA decreased from $21 (\pm 1)$ to $5.3 (\pm 1.4)$ and $11 (\pm 2)$ to $1.9 (\pm 0.8)$ min, respectively. This indicates that nuclease activity alone is not sufficient to describe the rapid clearance.

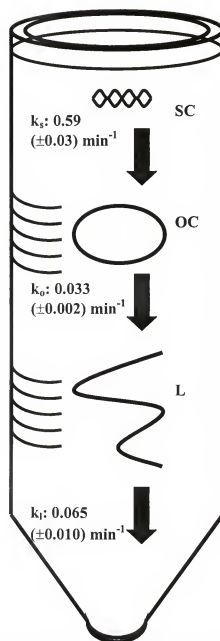


Figure 7-1. Schematic representation of pDNA degradation in isolated plasma

of pDNA from the bloodstream in rats

Chused and coworkers (Chused 1972) also suggested that nuclease activity was not enough to explain the rapid clearance of KB cell DNA from the circulation in mice. In this study, only 2 to 3 % of the radioactivity was hydrolyzed to trichloroacetic acid (TCA) soluble fragments in 30 min, which was several half-lives of the DNA in the circulation. Tsumita and Iwanga (Tsumita and Iwanga 1963) also found that less than 5 % of the total radioactivity was found in the TCA soluble fraction after 4.5 hours in mouse serum.

Alternatively, Gosse and coworkers (Gosse et al. 1965) suggested a major role for nucleases in the initial degradation of DNA after IV administration in rabbits and mice. This finding was based upon the proportionality between the initial rate of depolymerization and the plasma DNase activity level. Also, a rapid decrease in viscosity of isolated blood was discovered indicating a depolymerization of DNA. Finally a markedly slower disappearance of DNA-methyl green complex (a non-specific DNase inhibitor) than after native DNA.

The reason for this disparity in results deserved further investigation. Gosse utilized much higher doses of pDNA in their investigations, 200 µg versus 5 µg pDNA in Chused and coworkers 's investigations. This disparity may be due to saturation of a scavenger receptor, allowing nuclease activity to become increasingly important. The effect of increasing dose on the clearance of DNA deserved further investigation.

Effects of Increasing Dose of Plasmid DNA

The results presented in Chapter 4 reveal that all forms of pDNA (SC, OC, and L) are rapidly cleared from the circulation. Other investigators have qualitatively

commented on the rapid clearance observed after IV bolus administration of pDNA (Lew et al. 1995; Mahato et al. 1997; Thierry et al. 1997). However, these studies have been limited to the OC and L forms of the plasmid. The half-life of the SC topoform has been unable to be estimated due to lack of detection (Lew et al. 1995; Thierry et al. 1997).

The SC form of the plasmid was detectable only after the 2500 μg dose, and disappeared from the circulation after 1 min. One possible explanation for the rapid disappearance of SC pDNA is that it is rapidly being converted to the OC form *in vivo* by endogenous nucleases present in the plasma. If we compare the reported concentrations of DNase I in human plasma along with the reported SC pDNA nicking activity of DNase I under optimal conditions (Dwyer 1999) and make the assumption that the activity of rat DNase I is approximately the same as the human isoform (Takeshita et al. 1996), we can arrive at an approximate activity of 0.1 ng pDNA/ $\mu\text{l}/\text{min}$. This is far less than the *in vivo* SC pDNA nicking rate of 9.2 ng pDNA/ $\mu\text{l}/\text{min}$ observed 30 sec after administration of the 2500 μg dose. One minute after administration the *in vivo* rate was 2.7 ng pDNA/ $\mu\text{l}/\text{min}$. Thus, the activity of DNase I would not seem to be enough to describe the rapid conversion of SC pDNA to the OC form. The combined effect of enzymes in addition to DNase I is also insufficient to describe the kinetics in total. After IV bolus administration, the rate of degradation of SC pDNA was greater than 7 times faster than in isolated rat plasma.

The mechanism for the rapid clearance of SC pDNA may also be due to physical differences between the 3 forms. Previous studies comparing SC pDNA to L pDNA have shown that SC pDNA has stronger acidity in solution than L pDNA (Poly 1999). This difference is the result of the density and availability of the free phosphate groups.

Acidic phosphate groups located at the external loops of SC molecules would be available and involved in interactions, while most of the phosphate groups localized within the SC molecule would not interact with components in the bloodstream. OC and L pDNA however likely expose a much higher number of available acidic functional groups (Poly 1999). These anionic charges would be located all along the pDNA molecules and allow for multiple interactions. This decreased binding affinity of SC pDNA has been displayed in interactions with silica (Melzak 1996) and clay minerals (Poly 1999). The SC form of the plasmid has also been shown to interact more strongly with the hydrophobic stationary phase in reversed-phase high performance liquid chromatography (Colote 1986). This difference in exposed electrostatic groups could potentially explain the rapid clearance of SC pDNA relative to OC and L pDNA. If L and OC pDNA interact more strongly with plasma components than SC pDNA this may decrease their uptake by scavenger receptors or tissues. Furthermore, this association of OC and L pDNA with plasma components may also offer some protection from plasma nucleases. Protection from nucleases has been displayed after adsorption to proteins and is the basis for DNase I footprinting (Lodish 1995). This would result in SC pDNA remaining free in the bloodstream and open to nuclease digestion. Also, the increased hydrophobicity of SC pDNA may also lead to a greater interaction with vascular endothelia, providing an additional clearance pathway, and explaining SC pDNA's larger volume of distribution. Thus, these physical differences may provide some insight into the observed pharmacokinetic differences.

The OC pDNA displayed kinetics consistent with saturable elimination. Curve fitting to first order parameters also resulted in similar suggestions between doses. The

value of the first order rate constant for OC elimination ranged from 0.035/min to 0.14/min between the 2500 and 250 μg dose data sets, respectively. Thus it was necessary to include non-linear elimination into the model to describe this change in elimination between doses.

In conclusion, these results indicate that naked SC pDNA is cleared rapidly from the rat circulation after IV bolus administration at $390 (\pm 50)$ ml/min, and has a volume of distribution of $148 (\pm 26)$ ml. AUC analysis revealed that $60 (\pm 10)$ % of the SC pDNA appeared as the OC form of the plasmid. The OC form of the plasmid exhibits nonlinear characteristics with clearance ranging from $1.3 (\pm 0.2)$ to $8.3 (\pm 0.8)$ ml/min for the 2500 and 250 μg doses, respectively. Volume of distribution of the OC form was $43 (\pm 15)$ ml. The conversion of the OC form of the plasmid to the L form of the plasmid appears to be nearly complete. The L form of the plasmid is cleared at $7.6 (\pm 2.4)$ ml/min and has a volume of distribution of $38 (\pm 12)$ ml.

Results of the Curve Fitting Experiments

The model presented in Figure 5-1 successfully described the data. The values of V_{max} and K_m for OC pDNA remained relatively constant between doses of SC pDNA and also after administration of OC versus after administration of SC pDNA. After administration of SC pDNA, the average of all doses for V_{max} was $1.7 (\pm 0.5)$ versus $1.0 (\pm 0.3)$ ng/ μl /min after administration of OC pDNA. Likewise, the value of k_L remained relatively constant after administration of all 3 forms of the plasmid. The value of k_L ranged from $0.47 (\pm 0.11)$ to $0.45 (\pm 0.10)$ to $0.32 (\pm 0.02)$ min⁻¹ after administration of SC, OC, or L pDNA respectively.

Thus, we conclude from the modeling experiments that SC pDNA is rapidly converted to the OC form of the plasmid with a half-life of $0.15 (\pm 0.01)$ min. OC pDNA exhibits non-linear characteristics with a $V_{\max}$ of $1.5 (\pm 0.6)$ ng/ μ l/min and K_m of $6.0 (\pm 2.4)$ ng/ μ l. The L form of the plasmid exhibits first-order kinetics and is eliminated with an overall average half-life of $1.6 (\pm 0.4)$ min. Schematic representation of pDNA pharmacokinetic parameters after IV bolus administration of SC pDNA is presented in Figure 7-2.

Liposome: pDNA Complex Conclusions

These results indicate liposome complexation can indeed offer protection of the SC form of the plasmid. This increase in circulation half-life provides further evidence to support the use of liposomal delivery vehicles in gene therapy. This analysis also provides evidence that, although the liposome complexation does protect the SC form of the plasmid, the individual particles have an overall more rapid clearance from the circulation. The OC and L form of the plasmid also only remained detectable through 5 min after administration, versus through 20 minutes after administration of naked pDNA.

Liposome pDNA complexes were eliminated from the circulation more rapidly than the naked pDNA, while providing protection of the SC topoform from degradation in the bloodstream. This level of protection is independent of lipid: pDNA ratio from 1:1 through 6:1. SC pDNA is detectable through 5 min after administration as liposome: pDNA complexes at a 500 μ g dose, whereas it is undetectable after administration as naked pDNA at this dose. Clearance of the SC pDNA decreased from $390 (\pm 10)$ to 87

(± 30) after administration as naked or liposome complexes, respectively. Volume of distribution decreased from 148 (± 26) to 79 (± 16) ml after administration as naked or

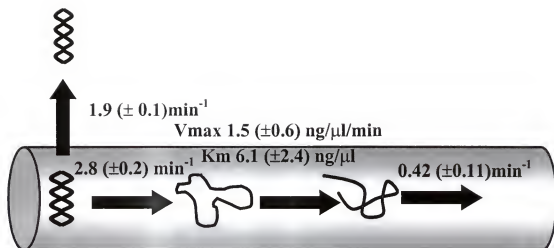


Figure 7-2. Schematic representation of pDNA pharmacokinetic parameters after IV bolus administration of SC pDNA in the rat.

liposome complexes, respectively. OC and L pDNA exhibited decreases in $C_{\max}$ after administration as liposome: SC pDNA complexes from $13 (\pm 4)$ to $5.8 (\pm 0.7)$ ng/ μ l and $3.2 (\pm 1.0)$ to $1.8 (\pm 0.8)$ ng/ μ l, respectively. Decreases in $t_{\max}$ after administration as liposome: SC pDNA complexes were also displayed for both OC and L pDNA from $1 (\pm 0)$ to $0.25 (\pm 0)$ and $5.3 (\pm 4.0)$ to $0.25 (\pm 0)$ min, respectively. Clearance/f increased after administration as liposome: SC pDNA complexes for both the OC and L forms of the plasmid from $3.0 (\pm 1.2)$ to $37 (\pm 9)$ and $6.9 (\pm 2.8)$ to $95 (\pm 37)$ ml/min, respectively.

These results indicate that liposome pDNA complexes are eliminated from the circulation more rapidly than the naked pDNA, while providing protection of the SC topoform from degradation in the bloodstream. This theory is schematically represented in Figure 7-3. This results presented here are consistent with previously published results (Osaka et al. 1996; Niven et al. 1998). Thus, liposome complexation may be an attractive means by which to protect the SC topoform, but the complex ability to target specific organs may be limited by their rapid clearance from the circulation.

Future Directions

The results and models presented here successfully described the pharmacokinetics of pDNA after IV administration in the rat. However, there are still a large number of studies that need to be performed. Among these directions, an important area is to link the PK model presented here to pharmacodynamic (PD) effect. Selection of an appropriate PD effect is not as direct of a relationship as for traditional drugs exhibiting receptor mediated effects.

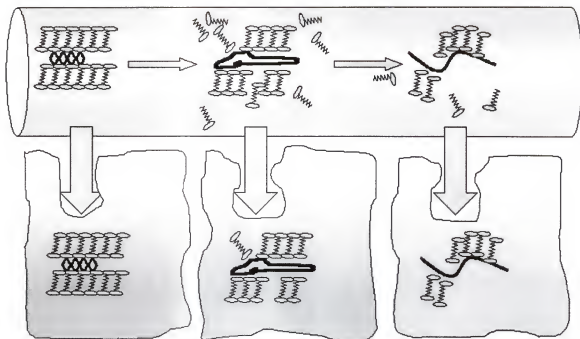


Figure 7-3. Schematic representation of liposome pDNA clearance from the bloodstream. In this model, removal from the bloodstream of the lipid: pDNA complexes is assumed to be larger than the degradation of the complex.

The most direct measure of the dose-response-over time relationship between pDNA and PD may be to measure the levels of mRNA transcript. This analysis could be performed by quantitative polymerase chain reaction. Using this method, levels of transcribed mRNA in various tissues could be calculated over time and correlated to the PK model presented here.

Alternatively, one could model the levels of transcribed protein in tissues over time as a PD parameter. This could be done by a number of methods including enzyme linked immunosorbent assay, western blotting, radio immuno assay, or immunoprecipitation. This measurement is a more direct relationship to clinical response. However, this relationship may be more difficult to obtain given that the translation into a final protein is a multistep process. It is likely that pDNA, mRNA, and protein stability in the cytosol differs between cells of various tissues and between cell types of a given tissue. Thus, correlation of protein levels may be more difficult to achieve. The kinetic processes within a cell will be an important area to be understood, in addition to kinetic processes in the bloodstream.

Furthermore, the application of this model may also need to be adjusted for different species. The clearance of DNA has been shown to be similar between strains of New Zealand, DBA/2, and BALB/c mice (Chused 1972), but differed between mice and rabbits (Gosse et al. 1965). Thus, this model may need to be adjusted not only for parameter values, but also in necessary parameters to describe the data.

Concluding Remarks

In conclusion, this work presents a pharmacokinetic analysis of pDNA *in vitro*, in isolated rat plasma, and *in vivo*, after IV bolus administration in the rat. Comparison of

the *in vitro* and *in vivo* results displayed that degradation by plasma nucleases was not sufficient to describe the pharmacokinetics of pDNA. In addition, the pharmacokinetic effects of liposome complexation were investigated *in vitro* and *in vivo*. These results displayed that liposome complexation was a means by which the SC topoform could be protected, but the complexes had an overall more rapid clearance from the bloodstream *in vivo*.

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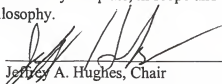
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BIOGRAPHICAL SKETCH

Brett Edward Houk was born on April 17th 1969 in Parma Ohio, and spent most of his childhood in Strongsville, Ohio. Brett attended the Ohio State University where he obtained Bachelor of Science degrees in both allied medicine and economics. After graduating from undergraduate studies, Brett moved to West Palm Beach, Florida and worked in Lantana Public Health Clinic in clinical pharmacokinetics. It was here that Brett decided to pursue a career as a pharmaceutical scientist. Brett was admitted to the University of Florida College of Pharmacy as a graduate student in August of 1996.

In his spare time Brett enjoys reading, swimming, working out, and playing sports of all varieties.

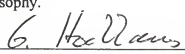
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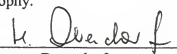
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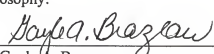
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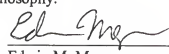
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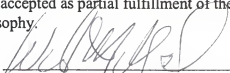


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